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NEGATIVE HORMONAL REGULATION OF SYMBIOTIC NODULE DEVELOPMENT. II. SALICILIC, JASMONIC AND ABSCISIC ACIDS
(review)

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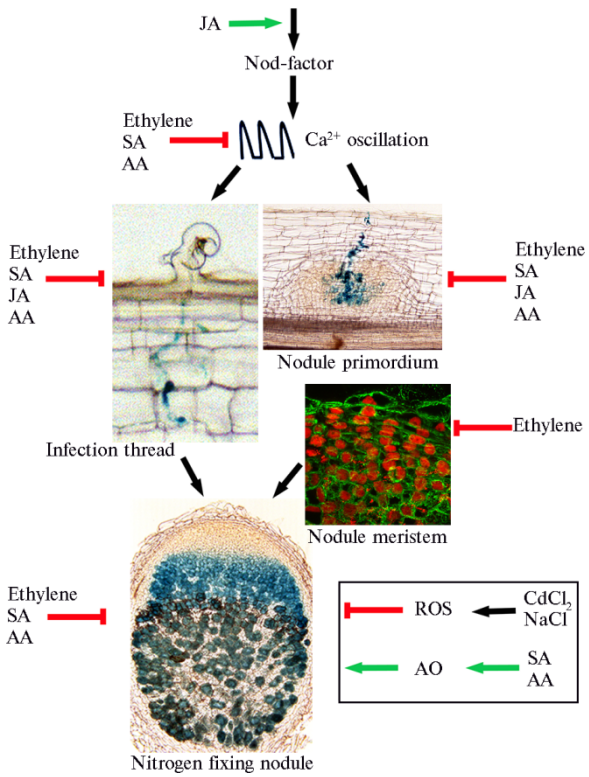
Abstract

As a result of interaction with rhizobia, legume plants are able to fix atmospheric nitrogen in symbiotic nodules. Development and functioning of symbiotic nodules are under strong host plant control, including phytohormonal regulation (B.J. Ferguson et al., 2014). Due to the fact that nodule formation is a highly energy-consuming process, nodule number is restricted by plant. The negative regulation of nodulation involves, along with ethylene (A.V. Tsyganova et al., 2015), also salicylic (P.C. Van Spronsen et al., 2003; G. Stacey et al., 2006), jasmonic (Sun et al., 2006) and abscisic (Ding et al., 2008) acids. It is important to note that all listed phytohormones act at the different stages of development and functioning of symbiotic nodules. The first negative effects of jasmonic and abscisic acids are related to the blocking of calcium oscillations (J. Sun et al., 2006; Y. Ding et al., 2008), induced by Nod factors (lipochitooligosaccharides synthesized by rhizobia and activating a program for the development of infection and nodule organogenesis). Calcium oscillations are also blocked by ethylene (G.E. Oldroyd et al., 2001). Salicylic, jasmonic and abscisic acids influence the further development of symbiosis, blocking both the growth of infection threads (through which the rhizobia penetrate into the root), and the formation of nodule primordia (T. Nakagawa et al., 2006; J. Sun et al., 2006; Y. Ding et al. 2008). For abscisic acid it was shown that its negative effect on the development of nodule primordia is mediated by the influence on cytokinin signal transduction pathway (Y. Ding et al., 2008). Salicylic, jasmonic and abscisic acids also negatively affect the nitrogen-fixing activity of the nodules, and for abscisic acid it has been shown that the negative effect is associated with the activation of the production of nitrogen monoxide NO (A. Tominaga et al., 2010). Nevertheless, all these phytohormones can have a positive effect on the formation and functioning of nodules. For example, jasmonic acid activates the expression of rhizobial *nod* genes that control the synthesis of Nod factors (F. Mabood et al., 2006). It is interesting to note that for salicylic and abscisic acids a positive role in activating the defense mechanisms in plants under the action of stress factors has been shown, which leads to a decrease in their negative effect on the functioning of the nodules (F. Palma et al., 2013, 2014). Future studies of the interaction of ethylene, salicylic, jasmonic and abscisic acids in the negative regulation of the formation of nitrogen-fixing nodules are of great interest. Such studies should shed light on why several phytohormones are involved in negative regulation and what the specificity of each of them is. It is important to study the possibility of practical use of mutants with a lower level of any of the phytohormones (ethylene, salicylic, jasmonic and abscisic acids). Therefore, it seems promising to study the mutant *enf1* (*enhanced nitrogen fixation1*), obtained on the model legume *Lotus japonicus* and characterized by an increased level of nitrogen fixation (A. Tominaga et al., 2009). At the same time, it should be considered that a change in the level of a certain phytohormone can have a negative impact both on the development of the plant and its response to abiotic and biotic stresses.

Keywords: plant-microbe interactions, legume-rhizobial symbiosis, symbiotic nodule, phytohormones

An adaptive capacity for biological fixation of nitrogen is characteristic feature of legumes as a result of interaction with soil rhizobia [1]. During the in-

teraction, specialized organs are formed on the legume roots, called symbiotic nodules; rhizobia in them are differentiated into bacteroids, which able to fix atmospheric nitrogen [2]. The symbiotic nodule development is based on the exchange of signaling molecules [3]. An important role in signaling belongs to phytohormones such as jasmonic acid [4], cytokinins and auxins [5, 6], gibberellins [7], ethylene [8, 9], abscisic acid [10], salicylic acid [11], strigalactones, and brassinosteroids [12-14]. The formation of a nitrogen-fixing nodule is very energy-consuming, that is why the nodule number is strictly regulated by the plant. We have previously reviewed the negative regulation of the development and functioning of symbiotic nodules by ethylene (Fig.).



Effects of ethylene, salicylic, jasmonic and abscisic acids on the development and functioning of a symbiotic nodule. The black arrows are subsequent stages of nodule development, the green arrows are the positive regulation, and the red arrows are the negative regulation. The box shows the regulation under the action of stress factors. JA — jasmonic acid, SA — salicylic acid, AA — abscisic acid, ROS — reactive oxygen species, AO antioxidants.

This review's focus is to discuss the role of salicylic, jasmonic and abscisic acids in the development and functioning of nodules of legumes. Certain specificity has been shown for these three phytohormones, although all of them affect negatively the development and functioning of nodules. In addition, jasmonic acid positively influences the very early stages of nodule development, and salicylic and abscisic acids have adaptive effect on the functioning of nodules under stress.

Salicylic acid is important in flowering, aging, resistance to pathogens and abiotic stresses [14]. The most studied role of salicylic acid in the induction of plant immunity when attacking pathogens. It is manifested in the activation of PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI), effector-triggered immunity (ETI), and in systemic acquired resistance (SAR) [15]. In recent years, the role of salicylic acid in the mutualistic legume-rhizobial symbiosis is particularly studied.

One of the first publications reported on the effect of different concentrations of salicylic acid on nodulation during the pre-treatment of *Vigna mungo* (L.) Hepper T-9 variety seeds. A decrease in nitrogenase activity was detected at all studied concentrations. At the same time, 10 μM concentration stimulated nodulation, whereas at higher concentrations (100 μM and 1 mM), the effect was negative [16]. In another study, the role of Nod factors in inhibiting salicylic acid-mediated defense responses in legumes was studied. Inoculation of alfalfa (*Medicago sativa* L.) by *NodC*$^{-}$ rhizobia mutant unable to synthesize Nod factors, as well as by the incompatible strain of *Rhizobium leguminosarum* bv. *trifolii* resulted in a signifi-

cant increase in salicylic acid accumulation compared to plants inoculated with a wild type strain [17]. Salicylic acid at 25 μM concertation, having no visible effect on plant development, caused a delay in the formation of nodules and a decrease in their number when used exogenously for pre-treatment of alfalfa plants before inoculation with rhizobia. Simultaneous addition of 10^{-7} M of Nod factor of *R. meliloti* and 25 μM salicylic acid to the nutrient medium reduced the number of formed primordia by 75 %. Consequently, Nod factors along with the induction of infection and nodule organogenesis are involved in suppressing the accumulation of salicylic acid, which is necessary for the successful development of nodule primordium [17].

Study of Frisson variety of pea (*Pisum sativum* L.) wild type plants and of P2 mutant (*sym30*) unable to form symbiotic nodules and arbuscular mycorrhiza showed salicylic acid accumulation in P2 plants inoculated with *R. leguminosarum* bv. *viciae* wild strain or the *NodC*⁻ mutant [18]. Inoculation with a strain of mycorrhizal fungus *Glomus mosseae* (Nico. & Gerd.) Gerd. and Trappe also led to a greater accumulation of salicylic acid in P2 plants compared to the wild type plants. In inoculation with a pathogenic *Pseudomonas syringae* ssp. *syringae* strain there were no differences in the amount of salicylic acid between the mutant and wild type plants. Perhaps the *sym30* mutation causes specific resistance to symbiotic microorganisms, which is associated with the accumulation of salicylic acid [18].

The negative effect of salicylic acid was also described for legume plants with determinate type nodules with limited activity of the nodule meristem. The treatment of soybean (*Glycine max* (L.) Merr.) seedling roots with 1 mM or 4 mM salicylic acid significantly reduced the number of nodules formed [19]. Treatment of the leaves with 1 mM salicylic acid, on the contrary, increased the nodule number. T. Sato et al. (20) compared the effect of salicylic acid on nodulation in the parental Williams soybean variety and in two NOD1-3 (rj_7) and NOD2-4 (rj_8) hypernodulating mutants forming increased nodule number. A 5-day-old seedlings were pre-treated with 100 μM salicylic acid for 5 days, after which they were inoculated with *Bradyrhizobium japonicum* USDA110 strain. There was a significant decrease in the number of nodules on the roots of the parental variety, and they were not capable of nitrogen fixation. At the same time, in both hypernodulating mutants, the nodule number was reduced much lesser. These results suggest that salicylic acid is involved in the autoregulation of nodulation [20].

The effect of salicylic acid was investigated in *Vicia sativa* L. plants inoculated by *R. leguminosarum* bv. *viciae* RBL 5523 and 248 strains. Salicylic acid at 100 μM concentration added to the nutrient solution completely inhibited nodule formation [21]. However, a 5 μM salicylic acid for strain RBL 5523 and 0.5 μM for strain 248 stimulated nodulation. In *Lotus japonicus* (Regel) K. Larsen plants inoculated with TONO, R7A and E1R strains and treated with 100 μM salicylic acid, nodulation was not inhibited. A negative effect of treatment with 100 μM salicylic acid was observed for pea plants of the parental variety Frisson and the supernodulating mutant P88 inoculated with *R. leguminosarum* bv. *viciae* RBL 248, for alfalfa (*M. sativa* L.) plants inoculated with *Sinorhizobium meliloti* RCR 2011, and for clover (*Trifolium repens* L.) inoculated with *R. leguminoarum* bv. *trifolii* ANU 843. At the same time, treatment with 100 μM salicylic acid did not inhibit formation of determinate nodules on bean (*Phaseolus vulgaris* L.) Negro Jamapa variety plants inoculated with *R. elti* CE3 and soybean plants (*Glycine soja* Siebold & Zucc.) inoculated with *S. fredii* HNO1 [21].

Subsequently, it was found that the negative effect of salicylic acid on *V. sativa* is manifested in the first 72 hours after plant inoculation. Pre-treatment for 24 hours did not affect the nodule number, and the addition of salicylic acid after 72 hours reduced their number by only 50 %. Salicylic acid has been shown to affect the association of rhizobia with the root surface, since curled root hairs and infection threads have not been detected. At the same time, root treatment with purified Nod factors caused deformation of root hairs under the addition of 100 μM salicylic acid, while cell divisions in the inner root cortex were blocked [21].

In further experiments, the transgenic *L. japonicus* and *Medicago truncatula* Gaertn. plants with bacterial *nahG* gene encoding salicylate hydroxylase were used [22]. Transgenic *L. japonicus* lines having one or two copies of the *nahG* gene had a decreased content of salicylic acid, which correlated with an increased number of nodules and infection threads in root hairs. Transgenic plants showed a significant increase in the root length. The authors did not note the increase in the number of infection threads per centimeter of the root compared to wild type plants. In experiments with spot inoculation of roots with rhizobia, which made it possible to level the effect of root elongation, it was shown that the nodule number increases in plants expressing *nahG*. Similar results were obtained with another model legume, *M. truncatula*. Inoculation of composite plants expressing *nahG* resulted in a 2-fold increase in the number of infection threads and nodules compared to the control, with no visible effect on root growth [22]. Probably, salicylic acid may be involved in plant autoregulation of the nodule number at the stage of infection thread formation through the activation of defense reactions [22] (see Fig.).

The role of salicylic acid in the nodule formation was also studied in cell cultures of wild type *L. japonicus* plants and the Nod^- mutant *Ljsym4-2* carrying a mutation in the *CASTOR* gene which encodes protein of nuclear membrane ion channel. This channel is involved in calcium oscillations induced by Nod factors [23]. The *Ljsym4-2* mutant is also unable to form arbuscules when inoculated with mycorrhizal fungus, as the fungal infection is aborted in the colonized epidermal cells in which the program of premature cell death is activated [24]. The mutant cell culture showed increased sensitivity to 0.5 and 1 mM salicylic acid, which was manifested in a sharp increase in the number of dead cells compared to that in the wild type cell culture [25]. A two-peak increase in the content of hydrogen peroxide was observed in the mutant culture, which is characteristic of the cellular response to the pathogenic attack [26]. Increased production of hydrogen peroxide preceded the activation of the production of nitrogen monoxide NO. That is, the content of salicylic acid, which wild type cells perceive as the physiological norm, in a mutant culture becomes a signal for the launch of cell death. This culture also revealed a constitutive increase in the expression of the *LjPR1* gene [25]. In composite plants, in the roots of which the *nahG* gene was expressed, no nodules were formed. This confirms that the altered sensitivity to salicylic acid in the mutant *Ljsym4-2* does not cause the Nod^- phenotype.

Two-day pre-treatment with 0.1 and 0.5 mM salicylic acid positively influenced nitrogenase activity and nodule biomass in 49-day-old alfalfa (*M. sativa*) plants which were subsequently exposed to 200 mM NaCl for 12 days [27]. It is suggested that this effect is associated with the antioxidant metabolism activation by salicylic acid (see Fig.). A positive effect was also observed when 10^{-5} M salicylic acid were used to treat aboveground part of 30-day-old chickpea (*Cicer arietinum* L.) variety Avarodhi plants which grew in soil contaminated with cadmium (25 mg) [28]. An increase in the nodule number, nitrogenase ac-

tivity, leghemoglobin content, as well as enzymes involved in nitrogen assimilation was observed in the control 90-day-old plants and those grown in polluted soil [28].

The study of *R. leguminosarum* bv. *viciae* 3148 strain revealed the presence in rhizobia of two systems of active efflux of substances from the cell (efflux pump) of the MFS (major facilitator superfamily) type controlled by the *salRAB* and *rmrA* genes activated by salicylic acid [29]. Mutation in the *salA* gene led to a significant inhibition of rhizobia growth in the presence of 2 mM salicylic acid, whereas alteration in the *rmrA* gene had no such effect. Both mutations did not affect the ability of rhizobia to form symbiotic nodules and their nitrogen-fixing activity. These results can be explained by the presence in the rhizobia genome of additional systems of active efflux of substances from the cell which compensate for the loss of the functional product of the *salRAB* and *rmrA* genes [29].

Jasmonic acid is involved in the regulation of various processes of plant development during biotic and abiotic stresses including legume-rhizobial symbiosis (30).

It was assumed that not only flavonoids, but also other substances associated with the phenylpropanoid pathway may be involved in activation of the rhizobia *nod* genes expression [31]. It has been shown that jasmonic acid and methyl jasmonate can induce the expression of *nod* genes in some strains of rhizobia, and jasmonic acid together with naringenin, a natural flavonoid inducer, have a synergistic effect [31]. Later, the positive effect of jasmonic acid and methyl jasmonate on the production of Nod factors (see Fig.) was confirmed for *B. japonicum* 532C and USDA3 strains [32]. The assumption that jasmonic acid, along with flavonoids, may be involved in the induction of rhizobia *nod* genes is also confirmed by the fact that its high content was observed in the root tips of soybean (*G. max* (L.) Merr.) seedlings of Williams variety [33]. It was also shown that jasmonic acid participates in the induction of the biosynthesis of flavonoids, since the treatment *of M. truncatula* seedlings with methyl jasmonate led to the induction of the *MtFNSII-2* gene encoding flavon synthase II [34].

However, in *M. truncatula* plants grown on a medium containing jasmonic acid the nodule number decreased. The effect was observed already at 0.1 μM concentration, and 10 μM concentration completely inhibited nodulation. However, the presence of 10 μM jasmonic acid in the culture medium did not affect the development of *S. meliloti*, that is, jasmonic acid primarily prevents the infection of host plant with rhizobia (see Fig.) [35]. Jasmonic acid has been found to suppress the expression of the *ENOD11* and *RIP1* genes that are activated at the initial stages of symbiosis, as well as calcium oscillations caused by the Nod factor [35] (see Fig.). It was previously shown that ethylene also blocks calcium oscillations [36] (see Fig.). High concentrations of jasmonic acid (100 μM) completely suppress calcium oscillations, while lower amounts change oscillation frequency [35]. In *sickle*, an ethylene-insensitive mutant, the frequency of calcium oscillations decreased at lower levels of jasmonic acid compared to the wild type, that is, ethylene inhibits the effect of jasmonic acid during the development of nodules and these hormones act as antagonists in the regulation of calcium oscillations induced by Nod factors [35]. Adding aminoethoxyvinylglycine, the ethylene inhibitor, to the wild type plants or using the *sickle* mutant significantly reduced negative impact on nodulation, indicating a synergistic effect of jasmonic acid and ethylene in regulation of nodulation (see Fig.).

Spraying *L. japonicus* shoots with 10^{-4}-10^{-3} M methyl jasmonate resulted in significant suppression of nodulation in wild type plants and the *har1-4* hypernodulating mutant. When plants were treated with lower concentrations of

methyl jasmonate (10^{-5}-10^{-6} M), a stronger inhibition was observed for the *har1-4* mutant, which may be explained by the higher effect of low concentrations of methyl jasmonate on the number of nodules formed in the mutant lacking the ability perceive the signal of autoregulation [37]. Methyl jasmonate has a negative effect on the nodule number, blocking the root hair curling, the growth of infection threads and the formation of nodule primordia [37] (see Fig.).

Jasmonic acid activates expression of *vspA*, *vspB* and *Lox2* genes in the hyper-nodulating soybean (*G. max* (L.) Merr.) SS2-2 mutant carrying a mutation in *NTS/GmNARK* gene which encodes a serine-threonine receptor protein kinase similar to CLAVATA1 [38]. In contrast, expression of the *PR1* gene controlling the response to salicylic acid was declined. The mutant also showed a 2-fold increase in the content of jasmonic acid in the leaves compared to the wild type. Treatment of plants with methyl jasmonate resulted in a decrease in the nodule number; however, in *har1-4* mutant the nodule number decreased to a greater extent than in the wild type plants [37], and in the SS2-2 mutant—to a lesser extent [38]. In not inoculated mutant plants, there was a higher expression of jasmonic acid response genes in the leaves but not in the roots compared to the wild type plants. *NTS/GmNARK*, as a negative regulator of jasmonic acid synthesis in the leaves, probably participates in a defense mechanism dependent on jasmonic acid, along with participation in the autoregulation of the nodule number [38].

In nodules of *M. truncatula*, allen oxide cyclase, an enzyme involved in jasmonic acid biosynthesis, is localized in plastids of uninfected cells in the nitrogen fixation zone, as well as in nodule cortex cells [39]. The pattern of distribution of allen oxide cyclase did not differ in nodules formed by effective and ineffective strains. Measurement of jasmonic acid level did not reveal a significant difference between the roots and nodules. In transgenic roots of the composite *M. truncatula* plants in which the *MtAOC1* gene was turned off by RNA interference (RNAi), there were no changes in nodule development, that is why the authors concluded that jasmonic acid does not participate in the regulation of this process [39].

However, jasmonic acid has positive effect on the formation of nodules in *L. japonicus*. The phytochrome B mutant (*phyB*) had a reduced content of photosynthesis products, as well as jasmonoyl-isoleucine conjugate, the active jasmonic acid derivative [40]. This mutant formed a smaller number of nodules, and 0.1 μM jasmonic acid increased their number, as compared to the wild type plants. In wild type plants grown at low the red/far red light ratio, the nodule number was also reduced, and jasmonic acid treatment increased this number. These data indicate that jasmonic acid is involved in the photomorphogenetic regulation of nodule formation through plant perception of the red/far red light ratio [40]. Incubation of 3-week-old soybean (*G. max* (L.) Merr.) nodules of the Don Mario variety for 5 days in solutions containing jasmonic or 12-oxophytodienic acid, a precursor in the biosynthesis of jasmonic acid, led to an increase in the number and size of cells in nodules [41].

Abscisic acid is an important plant hormone involved in the adaptation to various stresses such as drought, cooling, and salinity [42].

Treatment of pea roots with abscisic acid (see Fig.) inhibits nodulation and block cell divisions in the root cortex which are activated during the nodule formation [43]. The negative effect of abscisic acid has been shown for soybean (*G. max* (L.) Merr.) wild type plants and for hypernodulating mutant NOD1-3 [44, 45], as well as for the clover (*T. repens*) and *L. japonicus* plants [46]. Treatment of the *L. japonicus* plants with 1 and 10 μM abamine, the inhibitor of 9-

cis-epoxy-carotenoid dioxygenase which is involved in the biosynthesis of abscisic acid, resulted in a lower level of endogenous abscisic acid and an increase in the nodule number [46]. It was shown that abscisic acid significantly reduces the number of curled root hairs and hairs with infection threads [46] (see Fig.). Later, the negative effect of abscisic acid on nitrogen fixation was revealed. In pea plants, daily treatment of 3-week-old nodules with 100 μM abscisic acid for 9 days resulted in a significant decrease in nitrogen fixation [47]. The treatment of bean (*P. vulgaris* L.) seedlings with 1 and 10 μM abscisic acid also reduced nitrogen fixation. When 100 mM NaCl was added to the plants at 1 μM abscisic acid, nitrogen fixation was reduced to a lesser extent than without treatment [48]. Pre-treatment of alfalfa (*M. sativa* L.) plants with 10 μM abscisic acid reduced nitrogen fixation. Similarly to bean plants, treatment with abscisic acid reduced the negative effect of 200 mM NaCl on the nitrogen fixing activity of nodules [49]. The authors associate this effect with the activation of the antioxidant defense system in nodules by abscisic acid (see Fig.).

Experiments on *M. truncatula* plants revealed the dose-dependent negative impact of abscisic acid on the number of nodules and infection threads [50]. The negative effect was confirmed when analyzing the expression of two marker genes that are activated in the early stages of symbiosis, *RIP1* and *ENOD11*. Abscisic acid, like jasmonic acid [35] and ethylene [36], affects calcium oscillations induced by Nod factors (see Fig.). The 1 mM abscisic acid completely blocked calcium oscillations, whereas lower concentrations altered their frequency and amplitude [50]. It was established that at high concentrations of Nod factors, the negative effect of abscisic acid can be leveled, that is, during nodule development, the quantitative balance between Nod factor and abscisic acid is important [50].

Importantly, abscisic acid and ethylene regulate the development of nodules independently. In *M. truncatula* roots, the overexpression of the *abi1-1* mutant allele of the *ABSCISIC ACID INSENSITIVE1* gene of *Arabidopsis thaliana* (L.) Heynh. encoding nonfunctional protein phosphatase 2C, results in blocking the abscisic acid signaling pathway and an increase in the nodule number [50]. Another *sta-1* mutant for the *SENSITIVITY TO ABA* gene (*STA*) induced directly in *M. truncatula* formed decreased nodule number. At the same time, the sensitivity of formation of nodule primordia to abscisic acid was increased in the mutant, while the root hair deformation and curling under the action of Nod factors became less susceptible to abscisic acid [50]. It has also been shown that abscisic acid reduces the expression of *ENOD40* and *NIN* genes that is activated in the inner layers of the root during the formation of nodule primordium under the influence of cytokinin [50]. In *L. japonicus snf2* plants carrying a mutation with loss of function in *LOTUS HISTIDINE KINASE 1* (*LHK1*) gene which encodes a cytokinin receptor, abscisic acid suppresses the formation of spontaneous nodules. The obtained data confirmed the assumption that the abscisic acid blocks the development of nodule primordia by affecting the cytokinin signaling cascade [43, 50]. It was previously shown that the abscisic acid/cytokinin ratio is lower in the supernodulating soybean mutant *nts382* compared to the wild type [51].

Screening of *L. japonicus* mutants insensitive to abscisic acid revealed *enf1* (enhanced nitrogen fixation 1) variant with a 1.7-fold number of nodules and higher nitrogen fixation [52]. This mutant also produced more infection threads. It was shown that the mutant phenotype is determined by a reduced content of abscisic acid. Treatment of wild type plants with abamine led to higher nitrogen fixation accompanying a decreased amount of abscisic acid. The *enf1* mutation did not affect expression of genes involved in nitrogen fixation, but

caused a decrease in the content of nitrogen oxide NO [52, 53], the inhibitor of nitrogen fixation [54].

Thus, the negative effect of salicylic acid on the development of symbiotic nodules was revealed both during exogenous treatment and in transgenic plants when endogenous level of salicylic acid altered. The negative effect was observed in plants with determinate nodules with limited activity of the meristem and in plants forming indeterminate ones with long activity of the meristem. Salicylic acid also leads to a decrease in nitrogen fixation. It should be noted that under salt stress, the nitrogen fixation decreased to a lesser extent in plants subjected to salicylic acid pre-treatment, which is probably due to the activation of the antioxidant defense system when exposed to salicylic acid. The positive effect of salicylic acid on nodule formation and nitrogen fixing activity has been recently described in chickpea plants grown both in the presence and in the absence of cadmium salts in the soil. It was shown that exogenous jasmonic acid serves as a negative regulator of nodulation, and also can play positive role in induction of rhizobia *nod*-genes. However, endogenous jasmonic acid does not participate in symbiotic nodule development. Negative regulation of nodule development has also been demonstrated for abscisic acid using both exogenous treatments and mutants with an altered level of endogenous abscisic acid. Pretreatment with abscisic acid reduced the negative effect of salt stress, probably due to the activation of antioxidant defense.

Thus, in general, salicylic, jasmonic and abscisic acids, like ethylene, are negative regulators of the development and functioning of symbiotic nodules. Regulation occurs at different stages of nodule development. However, these phytohormones may be also involved in positive regulation, especially under stresses. Of great interest are further studies to describe the interaction between ethylene, salicylic, jasmonic and abscisic acids in regulation of legume-rhizobial symbiosis and to detect the peculiarities of the action of each phytohormone.

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METABOLOMICS AS A MODERN APPROACH FOR THE INVESTIGATION OF POTATO PLANT ADAPTATION TO BIOTIC AND ABIOTIC STRESS FACTORS

(review)

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Abstract

The progress in genomic and proteomic investigations has greatly expanded the range of subjects aimed in discovering of mechanisms involved in the regulation of plant growth and development under changing of environmental conditions. Another systemic biology approach, which is known as metabolomics, has almost the same significance. It focuses on the study of dynamics of low molecular compounds which results from the complex metabolic processes in the cell. The intensity of these processes is under the influence of both biotic and abiotic stress factors. Studies on metabolic analysis are carried out not only with model objects, but also with cultivated plants, including potatoes, listed among top 10 of the most valuable crops. This review aims to summarize the available literature data on systemic biochemical rearrangements detected with metabolic approach in potato under the action of pathogenic viruses and microorganisms, insects, as well as under the influence of abiotic stressors on potato plants. Recent data indicates that metabolic analysis allows characterization of the development and progression of viral and bacterial diseases, as well as testing resistance to the infections in various potato species and varieties (H. Hamzehzarghani et al., 2016; T. Stare et al., 2015; H. Tai et al., 2014; S. Tomita et al., 2017). Significant changes in a number of secondary metabolites are shown. The metabolic approach has sufficient sensitivity to detect also alterations under environmental stress. In the review, it was considered that the results of metabolic rearrangements of the potato cell are directly linked to dehydrogenation, including osmotic and temperature stressors. The changes in the content of amino acids and sugars are of particular importance. However, a number of additional studies are required for evaluation of shifts in potatoes metabolism which are triggered under the combined stress factors action, for example, desiccation and hyperthermia (V. Arbona et al., 2013; M. Drapal et al., 2017; R.D. Hancock et al., 2014). An absolute majority of the metabolic data was obtained with various vegetative organs of potato plants. Unfortunately, metabolic profiles of generative organs have not been studied yet. There is no information on the metabolic profiling of pollen formation, including CMS-forms of potatoes. This indicates the importance of this direction in the investigation of potato metabolome. Further standardization of the metabolic analysis and methods of result processing will make it possible to use the metabolomics not only as an important component of fundamental research, but in time, as a basis for monitoring of collection samples and newly created varieties and hybrids of potatoes. Analysis of modern data indicates their perspective for phenotyping of different potato genotypes, as well as for identifying forms that are resistant to various types of unfavorable conditions.

Keywords: metabolomics, *Solanum* spp., potato, biotic stress, pathogens, viral infection, fungal infection, pests, abiotic stress

Plants synthesize plenty compounds of various chemical structures. These compounds are generally grouped as those of the primary metabolism which ensure the existence of any living being, and of the secondary metabolism which are characteristic of certain (sometimes very limited) groups of organisms.

The number of plant metabolites exceeds 100,000, and only a small part of these substances are currently identified [1]. Substantially intensified research on their diversity became possible due the novel methods that allow characterization of all metabolites of a biological system, i.e. an organism, an organ, a tissue, etc. [2, 3]. Recently, gas chromatography—mass spectrometry (GC-MS), high-performance liquid chromatography—mass-spectrometry (HPLC-MS), ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS), capillary electrophoresis—mass spectrometry (CE-MS) and nuclear magnetic resonance (NMR) spectroscopy are used to separate and identify metabolites [4, 5]. Advanced technologies and uniform extraction and detection methods, and available databases for the identification of various compounds, as well as multivariate statistics methods provide excellent opportunities for comparing metabolite profiles, which is the basis of metabolomics.

Metabolite accumulation patterns determine significance of crops in the production of food and raw materials for the pharmaceutical, chemical and other industries [5]. Thence, metabolite data are not only of theoretical, but also of practical significance, and crop plants, in particular potato (*Solanum tuberosum* L.) cultivars, become models. Global annual yield of potatos reaches 300 million tons, which determines the role of potato cultivars for food security [6]. One of the first metabolomic studies on potato plants performed in 2000 using GC-MS [7] was focused on the analysis of carbohydrate metabolism compounds. The authors found differences between the tubers of plants grown in vitro and in the field conditions, as well as between wild type plants and transgenic lines [7]. Further metabolite profiling revealed differences between organs (for example, leaves and tubers responsible for the primary synthesis of carbohydrates and their deposition) and proved that metabolomics is a sensitive tool to evaluate genetic modifications and to control breeding [8].

The present review focuses on an analysis of current metabolomic approach to study mechanisms of adaptation to biotic and abiotic stressors of potato plants and to identify their resistance.

Effect of biotic agents. Plants that during growth interact with a huge number of organisms, including pathogens, have developed resistance mechanisms in the course of evolution. Omix technologies can trace manifestation of such mechanisms at different levels, i.e. from genome (differentiated gene expression, including the family of *PR* genes) through the proteome (production of protective proteins and enzymes for the synthesis of low molecular weight compounds) to metabolome (change in alkaloids, substances of phenolic nature, etc.) [1, 9]. In cultivated plants, secondary metabolites which often are the compounds involved in protection against infection, can significantly reduce the nutritional value of products or even are poisonous [10, 11].

Viral infections. Potato is infected by at least 40 viruses (http://www.kartofel.org/bolezn/virus/virus.htm). A large-scale studies revealed seven main types of potato resistance to viruses [12, 13], including resistance to infection (field resistance), resistance to virus accumulation, restriction of virus transport, resistance of mature plants, tolerance, resistance to vectors of viruses and hypersensitivity. Many plant systems at different levels, from cellular to organismic, are involved in each type of resistance. Recent data indicate that the virus attack initiates changes in the activity of a number of metabolic pathways, including carbohydrate metabolism and the synthesis of amino acids [14-16]. Consequently, metabolomics can be a tool to study mechanisms of resistance to viral infection in potato species and varieties.

Potato virus Y (PVY, *Potyviridae*) is among the most economically significant because of yield losses up to 80 % in susceptible varieties. PVY was first

described more than 80 years ago as an agent that causes degenerative processes in potatoes [17, 18]. The first data on modifications of the primary metabolism when exposed to the aggressive PVY^{NTN} isolate was obtained for potato varieties Desiree [19]. It was found that these processes are connected with the reproduction of the virus in the tissues. The authors studied predominantly carbohydrate metabolism and showed a correlation between a decrease in carbohydrate content and a change in the expression of genes encoding proteins of the photosynthetic apparatus and photoassimilation enzymes. In transgenic plants of the Desiree cultivar with overexpression of salicylate hydroxylase *NahG* gene from *Pseudomonas putida*, inhibition of photosynthesis was more pronounced and the amount of salicylic acid, a biotic stress hormone, lowered [19].

GC-MS metabolomic profiling identified 168 metabolites in dynamics during PVY infection in potato leaves and revealed differences between clusters associated with synthesis of amino acids and secondary metabolites, synthesis and degradation of cell wall substances [9]. Principal component analysis (PCA) showed clustering corresponding to the time of virus infection. The plant response to infection varied notably on day 3 and was more uniform on days 1 and 6. Analysis of variance (ANOVA) showed statistically significant changes ($p < 0.01$) for 83 metabolites, including 32 those identified, which depend on the stage of infection and PVY strain (PVY^N or PVY^{NTN}). PVY^N infection is asymptomatic, and PVY^{NTN} causes a mosaic, chlorotic and necrotic lesions of leaves, as well as tubers. In PVY^{NTN} infection, γ-aminobutyric acid (GABA), α-ketoglutarate, glycerate, maleate, maltose, phenylalanine, pyruvate, succinate, sucrose, and valine were varying metabolites. The amount of sucrose, glycerate, succinate and threonate decreased statistically significantly at the beginning of the disease in infected leaves as compared to the control ones. In PVY^N infection, the sucrose content also decreased significantly, despite the fact that this isolate is less aggressive. A similar dynamics was observed for most other metabolites involved in amino acid metabolism, the Krebs cycle, GABA shunting, and neutralization of reactive oxygen species (ROS) and phenylpropanoids. Valine and compounds associated with the metabolism of phenylpropanoids were found in infected leaves in larger amounts on day 6 when intensive reproduction of the virus occurred. PVY^N, in contrast to PVY^{NTN}, causes earlier accumulation of ROS neutralizing compounds in leaves, which is probably due to less damage caused by the less aggressive PVY^N strain [9].

The metabolomic changes are in line with the data of transcriptome analysis. The changes in carbohydrate metabolism, GABA shunting, phenylpropanoid and antioxidant metabolism depend on the period of the infection and the viral strain [9]. Accordingly, metabolic analysis is suitable for detection of viral infection and plant responses.

Fungal infections. Late blight. Phytophthora infestans is among the most harmful fungal pathogens of potatoes. This infection can lead to complete loss of potato crop [20, 21]. Fungicides which are used against late blight are quite expensive and negatively affect the environment. In addition, their regular use stimulates pathogen resistance. Growing varieties resistant to the pathogen is an alternative [22]. Potato plant resistance to late blight is controlled by many interacting genes and quantitative trait loci (QTL) some of which determine biochemical traits [23, 24]. Metabolomics can be a tool for describing biochemical changes and identifying markers of resistance in fungal infections [25].

Analysis of metabolite profiles of the aboveground parts of potato plants contrasting in susceptibility to phytophtore under controlled conditions show that phenylpropanoids, especially hydroxycinnamic acid amides, flavonoids, alkaloids and fatty acids the synthesis of which is more strongly induced after infection of resistant lines are primarily associated with resistance. During pathogenesis, ex-

pression of genes involved in the biosynthesis of hydroxycinnamic acid amides and flavonoids increased along with the change in the content of metabolites. These metabolites are known to be associated with antimicrobial activity and cell wall thickening (the latter helps prevent pathogen spreading in plant tissues from the site of infection and is considered a defensive reaction) [26-28].

In field conditions, metabolic changes in plants are due the combined action of various environmental factors. Thus, field trials are of an increased interest. In potato lines contrasting in resistance to phytophthora a comparative metabolic profiling of leaves by nuclear magnetic resonance (NMR) method with PCA data processing revealed clear clustering of samples collected during the onset of symptoms in susceptible forms [29]. Component loading showed that fatty acids, malate and rutin reaching higher levels in leaves of resistant varieties are resistance associated. Higher amount of succinate, by contrast, is characteristic of susceptible lines. Projection on the latent structures (PLS) method was also used to identify the relationship between the metabolite profile and the field resistance to the pathogen. The model developed for several varieties showed a high correspondence between the lesion described by the areas under disease progress curve (AUDPC) and the metabolite profile. VIP (variable importance in the projection) values showed that malate, rutin and sucrose play the greatest role in predicting the degree of the disease severity, the sucrose content positively correlates with AUDPC, and malate and rutin correlates negatively [29]. Metabolic analysis of samples collected in different stages of late blight led to the assumption that this approach can be reliable only when the profiles are used to compare dynamics over the course of infection. PCA method showed that the metabolite profiles of the samples collected at 12-day intervals are very different, and these differences are stronger than the inter-varietal variations. The authors indicated sucrose and malate as marker metabolites. Enzyme analysis of L-malate level to assess its relationship with late blight resistance confirmed the perspectives of metabolomics in assessment of plant resistance to pathogens and search for biomarkers of resistance [29].

A more detailed dynamic changes in the metabolism of potato tubers was studied in the unstable AC variety Novachip [30]. Analysis of polar and non-polar extracts revealed a total of 106 metabolites of which 95 were identified. Of these, 42 were attributed as pathogen-dependent, since their content significantly altered during late blight infection. The group of amino acids, including the precursors of secondary metabolites involved in defense against the pathogen, was subjected to the strongest changes. In Caesar and AC Novachip potato varieties having different resistance to phytophthora the metabolite profiling of tubers and leaves by GC-MS method revealed 77 compounds the content of which changed as the disease progressed [31]. Indole-3-acetonitrile, 3-hydroxybutyrate, D-mannitol, dihydrocoumarin and propionate were considered the protective metabolites, as their levels were much higher in more resistant variety Caesar. The spectrum of these compounds in the tubers and leaves is somewhat different, which may, according to the authors, indicate the features of the defense mechanisms in different organs. The authors also discuss prospects of new methods of metabolite analysis (e.g., NMR) in potato breeding for resistance [31].

Another paper reports on a close relationship of transcriptome and metabolome studies. RNA sequencing performed for the leaves of two resistant (F06025 and F06037) and one sensitive (Russet Burbank) potato genotypes revealed differences in the expression of 4216 genes in P. infestans infection [32], some of which encode enzymes involved in different metabolic pathways. Increased expression of these genes in resistant lines led to the accumulation of phenylpropanoids, flavonoids, alkaloids and terpenoids considered as protective

metabolites.

Rhizoctoniosis. Potato diseases caused by fungus *Rhizoctonia solani* attack the underground parts of the plant causing black scurf on stolons and tubers [33], which significantly decreases yielding [34]. A metabolite analysis by FT-ICR/MS (Fourier-transform ion cyclotron resonance) coupled with GC-MS method revealed peculiar metabolic pattern for infected potato shoots [1]. PCA analysis of 270 identified metabolites found differences between intact and *R. solani*-infected plants. PLS method also showed a clear difference between the metabolomes of healthy and affected seedlings. Mapping of infection associated alterations detected quantitative changes in the metabolites which are involved in 40 biosynthetic pathways and biochemically linked to 107 enzymes encoded by 222 genes [1]. Further search for marker metabolites indicated an increase in the activity of the mevalonate and deoxyxylulose phosphate pathways which led to activation in the biosynthesis of sesquiterpene pseudo-alkaloids (phytoberin, phytoalexins rishitin and solavetivon) and steroidal alkaloids with solasodine and solasodinin as aglycones. In infected seedlings, the content of most carboxylic acids, e.g. citramalate, oxalate, gluconate and α-keto-D-gluconate, was higher but pools of glucuronic and galacturonic acids, the cell wall components, decreased. In infection, the concentrations of substances potentially involved in systemic acquired resistance (SAR) mechanisms and hypersensitivity response (HR), such as azelaic acid and oxalic acid, were higher. The content of proteinogenic amino acids, except for pyroglutamic acid, was significantly lower in infected seedlings. Along with this, the pool of sugars in the infected shoots changed, viz. the content of D-fructose and myo-inositol decreased whereas the amount of other carbohydrates rose. The pathogen also influenced phenolic compounds in infected plant tissues, that is, the amounts of α-tocotrienol and ferulic acid increased while chlorogenic acid concentration decreased. At the same time, the content of amides of phenylpropanoids bound to the cell wall, in particular, N-feruloyl-tyramine, increased while the N-feruloylputrescine decreased [1].

Powdery scab. The disease of potato tubers and roots caused by *Spongospora subterranea* (Wallr.) Lagerh dramatically reduces plant productivity and tuber shelf life, which leads to significant economic damage. Root exudates in soil initiate germination of resting spores of the pathogen. Metabolomic analysis was used to identify marker compounds necessary for spore germination [35]. HILIC (hydrophilic interaction liquid) chromatography coupled with MS which mainly analyzes hydrophilic root exudates identified 24 low molecular weight compounds (mostly amino acids). Comparison of exudates of different potato varieties (Agria, Gladiator, Russet Burbank, and Iwa) revealed some peculiar features depending on the resistance of the variety to the pathogen [35

Thus, recent data indisputably show that different methods of metabolite analysis are good tools to study both progression of viral and fungal infections and resistance to viral and fungal diseases in plant species and varieties. Metabolite profiling of resistant potato varieties will facilitate potato breeding due to the use of biochemical markers.

Insect pests. The Colorado potato beetle (*Leptinotarsa decemlineata* Say), eating potato leaves, causes up to 30-50 % of crop losses [36]. Glycoalkaloids are a class of toxic metabolites of plant tissues, including potatoes, which protect plants from being eaten by herbivores. The main glycoalkaloids of *Solanum tuberosum* are solanine and chaconine [10], and the range of glycoalkaloids in other species of the *Solanum* genus is much wider [37, 38]. The leaf glycoalkaloids leptin and α-tomatin reduce the biomass eaten by adult beetles and increase the mortality of preimaginal larvae [39, 40]. In addition to alkaloids, many other metabolites are involved in

plant defense against Colorado potato beetles. For example, macrocypins, inhibiting cysteine proteases, interfere with the alimentation of herbivores [41]. Esters of some fatty acids and sugars are toxic [42], as well as sesquiterpenes [43].

Ultra high performance liquid chromatography coupled to quadrupole time flight spectrometry (UPLC-qTOF-MS) method used for leaf metabolite profiling in plants of potato wild species (*S. tarijense*, *S. oplocense*, *S. piurae*, *S. acroglossum*, *S. chomatophilum*, *S. paucissectum*) and varieties (*S. tuberosum*) with different Colorado potato beetle resistance elucidated the biochemical mechanisms underlying plant tolerance [44]. A search for metabolites associated with this trait showed that only *S. tuberosum* produces glycoalkaloids containing solanine and chaconine, the trisaccharide glycones. In the studied wild species, glycoalkaloids have tetrasaccharides in the side chains. In *S. oplocense*, *S. paucisectum*, *S. chomatophilum* and *S. piurae*, dehydrocommersonine, tomatine and neotomatine determine Colorado potato beetle resistance. A number of other metabolites that are not related to glycoalkaloids, for example, hydroxycoumarin and other phenylpropanoids, found only in wild species are also resistance-associated [44].

Sucking insects, including aphids, are another group of pests. Along with damage to the plant integuments and consumption of metabolites, these insects may often be the viral infection vectors. All this significantly reduces the potato yield [45, 46]. Unfortunately, to date, there is only one paper [45] on metabolite study with aphids on potatoes. The peculiarity of the work is that the metabolite profiles in leaves of different age, under attack of aphids and during viral infection were compared by NMR in intact and GMO lines. It was shown that the metabolite patterns change in all cases but to different extents. With genetic modification, the strongest metabolic differences manifest themselves in young leaves. The sucrose content significantly lowers whereas phenols and malate levels rise. The number of aphids eating on the plant does not differ between intact and genetically modified lines but depends on the content of glycoalkaloids which include α-solanine and α-hakonin. In young leaves, the accumulation of these metabolites is higher, which reduces the number of insects.

Thus, the metabolome analysis allows us to characterize the marker metabolites, the change in which underlies the response to lesions by leaf-eating and sucking insects.

In general, we can conclude that metabolite profiling, subject to more accessible equipment, can be a key approach in estimation of potato resistance to biotic factors.

Abiotic factors. Plant growth is largely determined by environmental conditions, i.e. physical and/or chemical factors. The light spectral composition, pool of macro- and microelements, temperature and water regimes can both accelerate and slow down plant development. Mechanisms underlying plant resistance to abiotic stressors at the transcriptome and proteomic levels is in focus of researchers since the 2000s, but metabolome studies are not numerous.

Drought and osmotic stress. Global warming leads to climate change, including a significant effect on the rainfall amount and duration. This may lead to droughts, salinization or flooding of agricultural lands. Plant resistance to moisture deficiency is the most studied. Accumulation of osmolytes (amino acids, sugars, polyols, etc.), antioxidants (glutathione, ascorbic acid, etc.) indicates a change in the intensity and direction of metabolic pathways [47], and therefore metabolite profiling is a tool to study the adaptation of plants to moisture deficiency [48].

It is known that potato plants are quite sensitive to the lack of moisture [49]. Metabolic rearrangements and their differences depending on drought resistance were compared in leaves, tubers and roots of the five potato genotypes

[50]. In the study, the authors used both non-targeted and targeted approaches. They examined about 7000 compounds in the first case and 60 in the second case, including the primary (glucose, malate, proline, etc.) and secondary (carotenoids, phenolic compounds, etc.) metabolites. It was shown that differences in metabolic alterations occur between organs and between genotypes. Stress-induced metabolic changes specific to each clone were analyzed by the targeted approach. The processes in the tubers were the focus. Organ-specific changes showed 45 compounds. In a lack of moisture, the accumulation of more complex phenolic compounds (naringenin, rutin and umbelliferone) is initiated in the leaves, but not in the roots and tubers. On the contrary, the content of their precursors and intermediates of the phenylpropanoid pathway (e.g. phenylalanine, chlorogenic and other phenylpropanoic acids) increased in all organs and in all genotypes, but with different intensity. Another group of metabolites accumulated in tubers is amino acids, e.g. glutamine, leucine, isoleucine, tryptophan, etc. However . the sugar level in tubers was almost independent of the stress factor, i.e. the changes mostly concerned only inositol and sorbose [50)].

Comparing leaf metabolites in Andean potato (*S. tuberosum* subsp. *andigena*) varieties Negra Ojosa and Sullu led to similar findings. These local varieties are more resistant to drought than varieties of cultivated potato *S. tuberosum* subsp. *tuberosum*. Metabolic profiling revealed a more pronounced accumulation of trehalose, proline and GABA in the less resistant variety Negra Ojosa at moisture deficit [51]. Concentrations of hexoses and complex sugars was practically unchanged and did not differ in both varieties, but in plants of more stable Sullu variety the level of organic acids of the Krebs cycle was higher during drought, which may indicate greater mitochondrial activity and stability.

The goal of a large-scale study of 31 potato varieties [52] was to search for drought resistance markers at transcriptome and metabolic levels by RNA sequencing and non-targeted metabolomic GC-MS detection. The minimum set of markers was 20 genes and metabolites. These allow prediction of drought resistance even at very early stages of cultivation. Interestingly, some markers were associated with resistance not only to drought, but also to pathogens, which suggests a commonality of resistance mechanisms to biotic and abiotic stressors.

Thus, metabolomic profiling can be a tool for phenotyping genotypes with different drought tolerance and study their physiological and molecular adaptations [50, 51].

With the use of an osmotically active sorbitol compound [53], two genotypes most contrasting in drought resistance were selected in vitro among two *Solanum* species and 18 varieties of *S. tuberosum*. Comparison of their proteomes under stressful conditions revealed different intensity of protein degradation combined with altered redox status [54]. Targeted metabolomic profiling of polar compounds detected changes for 26 of 42 metabolites examined in these two genotypes [53]. The authors evaluated the ratio of the amount of these compounds in experimental and control plants. A drought-induced decrease in the accumulation of metabolites occurred in most cases. Such dynamics was typical for ascorbate, aspartate, succinate, etc. The proline alteration is the most significant. In stress, proline accumulation was typical of both genotypes, but it was 11.39 times higher in the resistant ones. The accumulation of glycine, phenylalanine and sucrose was also noted. The obtained data are consistent with the dynamics of a number of earlier studied compounds [55].

Let us turn to another study in which metabolomic analysis was applied to assess tolerance to moisture deficiency in transgenic potatoes with a constitutively expressed *Arabidopsis* gene for transcription factor DREB that is induced during dehydration (DRE-protein protein/C-repeat binding factor; dehydration-

responsive element). DREB/CBF proteins bind to a specific site in the promoter and regulate gene expression during drought and low temperature [56]. A non-target metabolomic approach detected 165 metabolites (113 were identified) and revealed differences in tubers between control and transgenic plants. The most essential rearrangements in transgenic plants (i.e. higher accumulation) were characteristic of compounds involved in glutathione and GABA metabolism, which, according to the authors, indicates the activation of adaptation mechanisms due to the expression of the DREBA encoding gene. The presence of this protein can have both direct effect by changes in gene expression and an indirect effect, for example, by initiation of the ethylene phytohormone synthesis [56]. It should be emphasized that transgenic lines did not accumulate toxic solanine and chaconine. That is, an increase in the stability of transgenic lines did not lead to deterioration in consumer qualities of tubers. Thence, metabolic analysis is sensitive enough to detect differences in constructed potato lines.

High temperatures. Temperature is one of the most unpredictable abiotic factors affecting the growth and yield of potatoes [57]. Given that the potato was originally domesticated in the highland regions of the central Andes of South America, it is not surprising that these varieties are most productive in the temperature range of 15-19 °C [58]. In the future, due to climate warming, global potato production may decrease by 20-30 % [59], therefore, selection for resistance to elevated temperatures is becoming more relevant.

Genomic and transcriptome studies lead to the conclusion that resistance to elevating temperature is regulated by a large number of genes, changes in the expression of which affect biochemical and physiological reactions [57]. This generates more interest in studying the adaptation mechanisms of potatoes to rising temperatures at transcriptome and metabolome levels simultaneously (60). Significant rearranngements are shown for 89 of 123 detected metabolites. When hyperthermia decreased, the content of most of them diminished, including amino acids and nitrogen-containing compounds, such as ethanolamine and putrescine. The accumulation of the latter decreased both in leaves and tubers. The same trends were observed for organic acids associated with the tricarboxylic acid cycle, and for carbohydrates. The decrease in fumarate and succinate concentrations was especially pronounced in tubers. In leaves, a decrease in fructose, galactose and their phosphorylated forms was more intensive. A tuber specific change of metabolome with an increase in temperature affected the content of alcohols (decrease in the amount of sorbitol and mannitol, increase in inositol), lipid composition (higher saturation of fatty acids, greater amounts of C28-C30 fatty acids) and higher level of fatty alcohols, especially phytosterols.

The revealed metabolite rearrangements completely correlate with changes in the expression of genes encoding the enzymes of the corresponding biochemical cycles, which indicates the possibility of using the metabolomic approach to analyze biochemical rearrangements in potato plants influenced by abiotic stressors [60].

The available data indicate that the metabolomic approaches are sensitive enough to assess the effects of exposure to stressors directly related to cell dehydration (these changes mainly concern the composition of sugars and amino acids). The further studies will find out how metabolic rearrangements may be simultaneously influenced by several factors, for example, lack of moisture in combination with higher temperature.

Plant resistance to biotic agents is determined by immunity, including recognition receptors, the pathogen-associated molecular patterns, PAMPs, or microbe-associated molecular pattern, MAMPs) and effector-triggered immunity [13]. Resistance to abiotic factors is also based on highly specific recognition of

the stressor and on adaptive response, which can be divided into nonspecific and specific. A huge number of studies have shown that the basis of resistance is the activation of genes, including those responsible for quantitative traits (QTLs) [61]. In the present review, we have shown, using the example of potatoes, that the various metabolic reorganizations are dynamic processes depending on the type and strength of the stressor, as well as on the plant genotype. Adaptation to biotic and abiotic factors is provided by the accumulation of secondary metabolites and changes in the balance of amino acids and sugars.

Creating stress resistant potato varieties includes interspecific hybridization with wild-growing forms, cell engineering and genetic transformation [62]. Currently, along with genomic and proteomic technologies, metabolomic methods are being actively used in studying plant resistance and phenotyping wild and cultivated species, hybrids, varieties, and transgenic forms [8]. Recent strategy proposed to improve potato growing is based on the production of seeds of heterotic diploid hybrids (true potato seeds, TPS) [63]. Note that each of these approaches has its advantages and limitations.

In fact, all the data on potato metabolome have been obtained in the study of the vegetative organs of plants. The metabolome features of generative organs are currently not studied, there is completely no information on the metabolome profiles of CMS forms which are necessary for heterosis selection [64]. The available data indirectly indicate significant rearrangement in the metabolite profiles during the formation of pollen in *Solanacea* family, especially under the influence of elevated temperatures [65], which confirms the relevance of this trend in the study of potato metabolome.

So, metabolomic profiling becomes an integral part of basic research elucidating mechanisms of resistance to adverse biotic and abiotic factors. In addition, metabolomic analysis, after standardization of the analytical methods and data processing, can become a key element, and over time, the main technique to monitor potato plants in collections, created varieties and hybrids. There is no doubt that such studies are promising for the phenotyping potato genotypes, as well as for identifying forms that are resistant to various adverse effects.

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BIOLOGICALLY ACTIVE METABOLITES OF *Bacillus subtilis* AND THEIR ROLE IN THE CONTROL OF PHYTOPATHOGENIC MICROORGANISMS

(review)

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Abstract

The use of nonpathogenic soil bacteria living in association with the roots of higher plants enhances the adaptive potential of the hosts, stimulates their growth and serves as a promising alternative to chemical pesticides (V.K. Chebotar' et al., 2015). The bacterium *Bacillus subtilis* is recognized as a powerful biocontrol tool because of suppression of a wide range of phytopathogens due to the ability to produce a variety of secondary metabolites of different chemical nature, e.g. cyclic lipopeptides, polypeptides, proteins and nonpeptidic compounds (T. Stein, 2005). Information on the structure of bioactive metabolites of bacterial antagonists of phytopathogens, as well as mechanisms of their biological activity promotes targeted selection of strains for the development of microbiological products. *B. subtilis* is widely distributed due to the ability to form biofilms (A.L. McLoon et al., 2011). The chemical composition of compounds produced by the bacteria is determined by genetic characteristics and physical and chemical conditions of the environment. The cyclic lipopeptide surfactin exhibits antimicrobial (antibacterial, antiviral, antifungal) activity, causing lysis of the cell, and also contributes to a decrease in the production of mycotoxins by microorganisms (M. Mohammadipour et al., 2009). The structure of another peptide metabolite, rizocticin, promotes penetration into the microbial cell and inhibition of protein synthesis (K. Kino et al., 2009). *B. subtilis* can produce various hydrolytic enzymes which lyse the phytopathogenic fungus cell wall (C.P. Quardros et al., 2011). Among the metabolites synthesized by bacteria, lantibiotics play important role, their structure allows the synthesis of peptidoglycan which contributes to the formation of pores in cytoplasmic membrane (J. Parisot et al., 2008). A large family of polyketones exhibits antimicrobial activity due to the ability to collect multifunctional polypeptides into large pesticide complexes. The phospholipid antibiotic bacilizycin, which is produced immediately after the growth ceases and before the formation of thermostable spores, exhibits fungicidal activity against some fungi (A. Hamdache et al., 2011). Some strains of *B. subtilis* synthesize polyene antibiotics with conjugated double bonds, for example, hexaenes which inhibit growth of phytopathogenic fungi (E.B. Kudryashova et al., 2005). Several soil microorganisms, including strains of *B. subtilis*, can synthesize gibberellins and gibberellin-like substances that stimulate plant growth (R. Aloni et al., 2006). Proteins, lipopeptides, polysaccharides and other compounds associated with the *B. subtilis* cell wall can trigger the protective mechanism of the plant, that is, act as elicitors (M. Ongena et al., 2007). Thus, research aimed at studying biologically active metabolites of *B. subtilis*, which possess the properties of biopesticides or inducers of plant resistance to diseases, opens new prospects for the development of environmentally friendly technologies for protection against phytopathogens.

Keywords: biological control, *Bacillus subtilis*, metabolites, antimicrobial activity, biopreparation, phytopathogens, system resistance

Biological control by microorganisms is a promising alternative to an extend use of expensive pesticides, that accumulate in plants with adverse effects on humans health. Pesticides can also be lethal to beneficial soil inhabitants and cause emergence of pathogen strains resistant to fungicides. They have a short-term inhibitory effect of phytopathogenic microorganisms, whereas bioagents

affect negatively on phytopathogens during the whole growing season [1-5].

Non-pathogenic soil bacteria associated with the roots of higher plants enhance their adaptive potential of the hosts, and promote their growth. In 1980 J.M. Kloepper called them plant growth promoting rizobacteria (PGPR). One of the plant rhizosphere characteristics, reflecting its colonization by microorganisms, is a rhizosphere/soil quantitative parameter (R/S). In most rhizobacteria, the R/S value varies from 2 to 25 [6]. Rhizobacteria can act as biocontrol agents due to ability to compete with phytopathogens for ecological niche [7], to produce different antibacterial compounds [8-10], to affect plant defense system, to promote plant growth by increasing availability of nutrients (nitrogen, phosphorus, amino acids) from soil [11].

The purpose of this paper is to data about biologically active metabolites of *Bacillus subtilis* which is recently considered a powerful biocontrol tool.

B. subtilis can contact with higher plants and promotes their growth. *B. subtilis* species is better than other agents of the genus *Bacillus* and more suitable as biocontrol agent because of host wide range, ability to form endospores and to produce different antibiotics [12]. *B. subtilis* has suppressive activity in vitro against more than 20 phytopathogens due to the ability to produce a variety of secondary metabolites, e.g. cyclic lipopeptides, polypeptides, proteins and non-peptidic compounds [13, 14]. These agents, mainly peptides, are of ribosomal or non-ribosomal origins [15].

The main antibiotics of *B. subtilis* which suppress phytopathogens are peptide derivatives, mainly lipopeptides, synthesized non-ribosomally [15]. Lipopeptide antibiotics are produced by binding β-hydroxyl residues or β-amino groups with fatty acids. The length and branching of fatty acids chains and amino acid redisues determine the product properties [16].

B. subtilis bacteria are common in the environment, because many wild strains are able to form biofilm on the plants roots surface [17, 18]. Cyclic lypopetide surfactin contains carboxylic acid (3-hydroxy-13-methyltetradecanoic acid) and seven aminoacids. The molecule contains heptapeptide associated with the β-hydroxy fatty acid through lactonic bound [19, 20]. Another surfactin analogues are pumilacidin, bacircin and lihenizin [21]. Surfactin is one of the most active biosurfactants [13, 21], famous as simulator of biofilms formation. Partly, it is due to activation of membrane-sensitive histidine kinase [17, 22, 23].

Exopolymeric compounds play an important role in biofilm formation, and their chemical composition affects biofilm properties and quality [24, 25]. Biofilms promote the colonization of roots by bacteria and thereby increase the local antibiotics concentration [26]. Also its formation enhances antimicrobial resistance [27-29]. Surfactin has antibacterial, antiviral, antifungal, insecticide, herbicidal activities [30-34], stimulates resistance to the pathogen penetration affecting on protective plant mechanism [35, 36]. Biocontrol of the phytopathogenic fungus *Aspergillus flavus* by surfactin reduces plant contamination by mycotoxins (37).

Many authors group mycosubtilins, iturin and bacillomycin, the cyclic lipoheptapeptides which are similar in structure and show powerful antifungal and hemolytic features but limited antibacterial activity, under the general title iturins. Antifungal effect is manifested in interacting with the cytoplasmic cells membrane with formation of ion-permeable pores [38, 39]. In China, a new strain of *B. subtilis* has been isolated, which can produce jiean-peptide, an antibiotic similar in structure to iturin [40]. Jiean-peptide manifests fungicidal properties against various plant pathogens [41, 42]. The strain can produce this biofungicidal compound provided that the bacterial cells are adsorbed on wood pieces.

Fengycin (synonym plipastatin) combines several compounds of unusual

structure, i.e. cyclic, branched components and rare substances [43]. It contains β-hydroxy fatty acid associated with the N-terminal decapeptide, which includes four β-amino acids residues and rare L-ornithine amino acid. The C-end residue of the peptide is partially linked with the tyrosine residue at position 3, with branching point of acyl peptide and the 8-membered lactone ring also persisting [15]. Fengycin has antifungal activity against some thread like fungi [44]. This compound is successfully used to control *Fusarium moniliforme*, due to inhibition of mycelia growth and spore formation. A possible mechanism of fungicin antifungal activity involves interaction of the styrene molecules and phospholipid membrane which disrupts target cell membrane structure [45-47].

Rhizocticin is a phosphonate oligopeptides antibiotic produced by the gram-positive *B. subtilis* ATCC 6633 strain [48]. This is di- and tripeptide, containing arginine amino acid and L-2-amino-5-phosphono-3-pentenoic amino acid, not found in proteins. Rhizocticins penetrate into the fungal cell through the oligopeptide transport system. As a result, the non-protein phosphate-containing amino acid peptidase releases which inhibits protein synthesis. Phosphonate compounds are common among biologically active substances mainly due to their ability to influence the carboxy- and phosphate-containing metabolites [46].

Lantibiotics (lanthionine-containing peptide antibiotics) are ribosomally synthesized peptide antibiotics with unique features. Lanthionine is produced by ribosomal synthesis or by modification (serine dehydration and subsequent binding with thiol cysteine groups) [49]. Properties of various types of lantibiotics depend on their structure and, thence, differ. Lantibiotics of A type (21-38 amino acid residues) have more linear secondary structure and destroy gram-positive target cells, forming pores in the cytoplasmic membrane.

Subtilin is a 32-amino acid pentacyclic lantibiotic structurally similar to nisin of *Lactococcus lactis* which is widely used in biocontrol [50]. Both cell density and sporulation can regulate synthesis of lantibiotics. The lantibiotics produced by gram-positive bacteria inhibit synthesis of peptidoglycans and shorten the peptidoglycan molecule, which facilitates the pores formation [51]. Serine proteases also participate in the subtilin synthesis. High lipopeptide mycosubtilin content (880 mg/g) is found in the *B. subtilis* strain with antagonistic effect on *Candida* sp. [18].

Ericin S differs from subtilis only in four amino acids, that is, the antimicrobial properties of both lantibiotics should be comparable. However, ericin A differs from erysin S in the ring structure and the position of 16 amino acids [16]. The lantibiotic mersacidin refers to type B lantibiotics which have a larger molecule size and a diverse structure.

Subtilomycin is synthesized by *B. subtilis* MMA7 isolated from the marine sponge *Halilona simulans*. Several strains of *B. subtilis* synthesize subtilosin A which has a macrocyclic structure with three intermediate bounds, including both ether bonds between cysteine sulfate and α-carbon of amino acids [15]. Sublancine 168 with β-methyllanthionine bridge and two disulfides bonds rare for lantibiotics is active mainly against gram-positive bacteria.

B. subtilis bacteria are applied as producers of amylase, protease, chitinase, xylanase, lipase, gluconase, cellulase, and other enzymez [52, 53]. Bacilli attach to hyphae and lyse fungal cell walls to use lysates as an additive nutrients and energy sources [54].

B. subtilis along with the peptide antibiotics produce polyketones which are active agents for phytopathogens biocontrol. Polyketones are a metabolite family which consists of polyketon synthetase enzymes with antimicrobial activity due to ability to gather multifunctional polypeptides into big pesticide complexes. These are linear molecules with two amide bounds and different residues

and substituent in structure. These metabolites are grouped based on structure and functions [40, 43].

Phospholipid antibiotic bacilysocin is produced by *B. subtilis* 168 just after growth cessation and before thermostable spore formation. Its activity is more pronounced against eukaryotic *Sacharomyces cerevisiae*, and also lower fungi *Candida pseudotropicalis* and *Cryptococus neoformans* characterized by non-filamentary growth [55, 56].

Phospholipids produced by *B. subtilis*, possess antimicrobial activity against gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*), gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), *Actinomyces* sp. and fungi (*Aspergillus niger, Candida albicans*) [57]. It has been found, that their antimicrobial effect is enhanced with rising temperature (up to 50 °C) and pH (up to 10) [58, 59].

Some *B. subtilis* strains produce metabolites of polyene antibiotic groups with conjugated double bonds. Hexaenes of other *B. subtilis* strains inhibit the growth of phytopathogenic *Fusarium culmorum*, *F. sporotrichiella*, *F. oxysporum, Botrytis sorokiniana*, *Alternaria tenui* and *Phytophthora infestans* [60].

Isocoumarins are phenolic compounds that occur in *Bacillus* species as phenylpropanol derivatives. Eleven strains of *B. subtilis* isolated from various geographical and ecological niches, produce amicoumacins classified as antibiotics of the isocoumarin group. Amicoumacin and bacillosarcin extracted from the culture liquid of *B. subtilis* marine bacterium TP-B0611, protect plants from grain moth [43].

Isoprene makes the smallest group of the natural terpenoids. Unlike others bacteria, *B. subtilis* 6051, *B. subtilis* 23029 and *B. subtilis* 23856 produce volatile isoprene at relatively high concentrations [43]. Sporulenes A, B and C are three terpenoids isolated from *B. subtilis* spores, which can protect spores of bacilli from oxidative stress. The biological role of sporulenes is determined by sporulation of *B. subtilis* [43].

Some strains of *B. subtilis* produce gibberellins and gibberellin-like substances [61]. Cytokinins are regulators of cell division and differentiation in various plant tissues. They play an important role in the growth and nodules formation. It is shown that *B. subtilis* cells produce volatile compounds stimulating plant growth, mainly of 3-hydroxybutan-2-one and butane-2,3-diol [46, 57].

Induced plant resistance is due to interaction between plants and microorganisms among which bacteria of rhizosphere, in particular *B. subtilis*, play an important role. Proteins, lipopeptides, polysaccharides and other compounds associated with the cell wall of the *B. subtilis* may be elicitors which trigger plant defense response [61, 62]. Bacterial metabolites trigger cascade of defense processes, including formation of reactive oxygen species, proteins phosphorylation, initiation of phytoimmunity mechanisms, that lead to the development of system resistance [63, 64]. Cyclic lipopeptides, the surfactin, iturin, and fenghin, can influence the signaling cells of plants, that results in initiation of natural immune responses [65].

Compounds released from the cell wall of the phytopathogens by hydrolases of antagonists can function as elicitors of resistance and cause defensive response, e.g. synthesis of phytoalexins, activation of hydrolytic enzymes, lignification etc. For example, *B. subtilis* AF1 strain isolated from the soil suppressive to *Fusarium udum* can induce resistance against *Aspergillus niger* in ground-pea [66]. This strain, being an inducer of resistance, is a stimulant of accumulation of phenylalanine ammonia-lyase and peroxidase. In other systems, significant changes of plants cells defense responses are related to phenol modifications. Treatment of tobacco plants with cell suspension at low surfactin concentration

activates phosphorylation and oxidative reactions, leading to plant cell death and penetration of phytopathogens [67].

So, *Bacillus subtilis* cells produce significant quantity of bioactive metabolites, having different chemical structure: cyclic lipopetides, proteins, polypeptides, ketone, polyenoic compounds etc. Ability to synthesize compounds of a particular structure presumes a specific mechanism of bacterial action on a phytopathogenic organism, and also explains the biological activity of a particular bacterial strain against certain microorganisms. When selecting effective producer strains, it is necessary to pay attention to the structure of their metabolites, since they can be the basis for the development of environmentally friendly technologies for plant protection against phytopathogens.

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FULLERENE DERIVATIVES INFLUENCE PRODUCTION PROCESS, GROWTH AND RESISTANCE TO OXIDATIVE STRESS IN BARLEY AND WHEAT PLANTS

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Abstract

Creation of effective environment-friendly preparations to improve productivity and sustainability of agro- and ecosystems is of current interest. Carbon nanostructures, such as the water-soluble C_{60} and C_{70} fullerene derivatives presently used in biomedicine and pharmacology, are considered perspective agents for agriculture. It was shown that they can penetrate into the cell membranes owing to lipophylicity and nanosize, transport medicinal substances to target cells and have antioxidant activity. The mechanism underlying the influence of water-soluble fullerene derivatives on plants in agroecosystems remains unclear. In the paper, we for the first time report the effects of C_{60} fullerene derivatives on the processes that determine the net productivity and plant resistance to oxidative stress. In the study we used fullerenol and the fullerene C_{60} adducts with the three essential amino acids, threonine, lysine, arginine, and also with the amino acid hydroxyproline, which were previously synthesized following a one-step procedure. Stimulating effects of these fullerene derivatives on the growth of spring wheat and barley were observed in two vegetation experiments carried out in controlled conditions (aerated nutrient solution, plant growing light equipment) when the compounds were added to the root habited medium and under non-root treatment. It was shown that the biomass of leaves, stems, and roots in plants increased by 27-226 % ($p < 0.05$). Statistical analysis using the Wilcoxon test confirmed the reliability of the differences found. Fullerenol, fullerene C_{60}-hydroxyproline, and fullerene C_{60}-threonine caused the greatest increase when compared to the control. Obviously, the observed effect was associated with the established ability of fullerenol and C_{60} fullerene amino acid derivatives to exert regulatory activity on the synthesis of photosynthetic pigments and, as a consequence, on the efficiency of photosynthesis. A comparison of the reflection indexes characterizing the content of chlorophylls (ChlRI) and anthocyanins (ARI) in leaves showed that the photosynthetic apparatus with a greater potential is generally formed under the in-

fluence of fullerene derivatives. Under the influence of these derivatives, the lipid peroxidation intensity also decreased and superoxide dismutase was activated while reactive oxygen species generation in leaves and (or) roots increased (predominantly in barley) or decreased. These changes in plants were the most expressed at fullerenol, C_{60}-threonine and C_{60}-hydroxyproline action. Under stress modeling (UV-B irradiation, 20 kJ/m^2), the UV-resistance of barley plants after not-root treatment with fullerenol, C_{60}-threonine and C_{60}-hydroxyproline, when estimated by the dry weight of the above ground parts and roots, was 10-20 % higher compared to that of the control irradiated plants which were of less weight (by ≈ 33 % for stems and leaves, and by 10-20 % for roots). Thus, the study revealed the positive influence of synthesized amino acid derivatives of fullerene C_{60} and fullerenol on the plant production process and resistance to oxidative stress. High efficiency in small concentrations, low expenses for application and environmental friendliness indicate the perspectiveness of these compounds and necessitate further studying the mechanisms of their action on the soil—plant system to create preparations for use in plant growing.

Keywords: amino acid fullerene C_{60} derivatives, fullerenol, plant production processes, optimization, oxidative stress, resistance, ecologically safe preparations, plant growing

The requirement for biodegradable adaptogens and protectors stimulates the development of innovative forms that provide transportation of macro- and microelements and physiologically active compounds to plants. Prospects in this field are associated with carbon nanomaterials, in particular with water soluble fullerene derivatives [1, 2]. The high lipophilicity of the carbon core of fullerene derivatives ensures their penetration into biomembranes [3-5], the nanoscale size provides a steric conformity to biomolecules, and the π-electrons cloud on the surface allows participation in free radical processes which can be multidirectional depending on the fullerenol concentration, features of the impact object and conditions [3, 6, 7]. Most water-soluble fullerene derivatives are compounds with functionalized hydroxyl, carboxyl and amino groups [1].

Peculiarities and mechanisms of water soluble fullerene derivatives' influence on plants in agro- and ecosystems are poorly studied, since these researches only begin to develop extensively [4, 6, 8, 9]. The water-soluble derivatives are reported to have effects of both inhibition of growth and stimulation of growth, development and productivity. Thus, poly(oxy)hydroxylated fullerene damaged onion cells [10], but promoted the growth of the arabidopsis hypocotyls and green alga *Pseudokirchnerella subcapitata* [6]. Treatment of bitter melon seeds with a solution of poly(oxy)hydroxylated fullerene led to 54 % increase in plant biomass, 128 % increase in yield, and 90 % higher content of useful substances [8]. The positive impact of these compounds on plants is presumably associated with antioxidant activity, such as the ability to bind reactive oxygen species [11-13].

Earlier we reported on the ability of fullerenol C_{60} to prevent oxidative stress in roots of cereals and subapical root thickening after UV-B irradiation of seedlings due to a decrease in the reactive oxygen species (ROS) content. The results of treatment of tested plant seeds (spring barley *Hordeum vulgare* L.) with different concentrations of fullerenol C_{60} revealed its high biological activity [9].

Absorption, translocation and accumulation of C_{60} or C_{70} fullerene derivatives are described in rice, radish, onions, bitter melon, and wheat [8, 10, 14-16]. Water soluble fullerene C_{60} derivatives penetrate through animal and plant cell membranes as lipophilic ions or in neutral form after protonation [4]. On seedlings of wheat (*Triticum aestivum* L.) and radish (*Raphanus sativus* L.) it was shown that the uptake of fullerene derivatives C_{60} and C_{70} by plants depends on fullerene concentrations in the root area and that these compounds are accumulated mainly in the roots [15, 16].

Almost nothing is known about the possible mediated influence on plants of water soluble fullerenes derivatives after they get into the soil. We have shown for the first time that an increase in net productivity and resistance of plants to

oxidative stress after the amino acid C_{60} derivatives and fullerenol introduction into the root area or foliage treatment is obviously associated with the established changes in the structure and efficiency of the photosynthetic apparatus and also with the influence on the antioxidant protection system, namely, the intensity of lipid peroxidation, the activity of superoxide dismutase, and the generation of reactive oxygen species.

The aim of the research was to evaluate fullerene C_{60} derivatives impact on the production process and plant resistance to oxidative stress under controlled conditions.

Techniques. Water soluble C_{60} derivatives (fullerenol and C_{60} adducts with L-lysine, L-threonine, L-arginine and L-hydroxyproline amino acids) were obtained by previously developed a one-step synthesis method from individual fullerenes, fullerene mixture or fullerene soot with the use of alkali water solution and an interphase catalyst (TBAH) [17, 18].

The effects of fullerene derivatives on Leningradskaya 6 spring soft wheat (*Triticum aestivum* L.) and Leningradskii and Belogorskii spring barley (*Hordeum vulgare* L.) varieties were studied under controlled favorable conditions and under oxidative stress simulated by UV-B irradiation of the aerial parts of the plants (APHI bio-polygon). Plants were grown in vessels with aerated nutrient solutions, under artificial illumination [19] for 1 month (until appearance of leaves 6-7, stem elongation stage). The light installations equipped with elevating blocks of DHa3-400 lamps (Russia), lighting intensity was 80-90 W/m^2 for photosynthetically active radiation (PAR) with 14 h light period, the air temperature was 25±2 °C at 65±5 % relative humidity. Ten plants were used per vessel, with repeatability of 30 plants per test variant. The nutrient solution in the vessels was aerated permanently, replacement of the solution and pH control were carried out every 3 days. The macro- and microelements composition has been proposed earlier [9].

Synthesized fullerene C_{60} derivatives were introduced into the root area (into aerated nutrient solution) [9] at 1 mg/l concentration or by foliar application (0.1 mg and 15 mg per 1 l of macro- and microelements solution) [9]; the concentrations of the derivatives are based on preliminary research data. The macro- and microelements solution without fullerene derivatives was the control [9]. The pH values of tested solutions were within 6.2-6.9 (the variation of pH in this range does not have a significant effect on plant growth). In variants with foliage application of C_{60} derivatives with macro- and microelement solutions (nanocompositions), plants were sprayed 3 times per vegetation (with 7 days periodicity during the tillering—stem elongation); plants treated with macro- and microelements without C_{60} derivatives were the control.

To create stressful conditions, the plants were exposed to UV-B radiation during stem elongation stage. Before irradiation, the plants were treated 3-fold (7 days periodicity) by foliage application of nanocompositions of fullerene derivatives. Irradiation of the plants started 3 days after the last foliage treatment with nanocompositions. The dose of biologically effective UV-B radiation was 20 kJ/m^2; LE-30 lamps (Russia) with a spectral range of 280-380 nm and maximum intensity at λ = 320 nm were used. Ten plants were used per vessel, with repeatability of 50 plants per variant. Non-irradiated plants treated with macro- and microelements without C_{60} derivatives were the control.

Antioxidant properties of fullerenes were assessed via lipid peroxidation intensity determined by accumulation malonic dialdehyde (MDA), superoxide dismutase activity (SOD) in nitroblue tetrazolium test (by ability to compete for superoxide radical), and generation of reactive oxygen intermediate (ROI) estimated by conversion of adrenaline to adrenochrome which optical density was

measured at λ = 480 nm [20-22].

The parameters of leaf reflectance spectra were registered in the range from 400 to 1100 nm with 0.3 nm increments (a fiber-optical spectroradiometric system, Ocean Optics, Inc., USA) at 0.065 nm resolution [23]. The main elements of the system are a spectrometer HR2000, SpectraSuite software, tungsten halogen light source LS-1 (standard), Spectralon standard WS-1, standard probe of reflection/backscattering R-200-7-UV-VIS. Indexes of reflection were calculated to quantitate chlorophylls, flavonoids, anthocyanins in leaf tissue and other photosynthetic parameters.

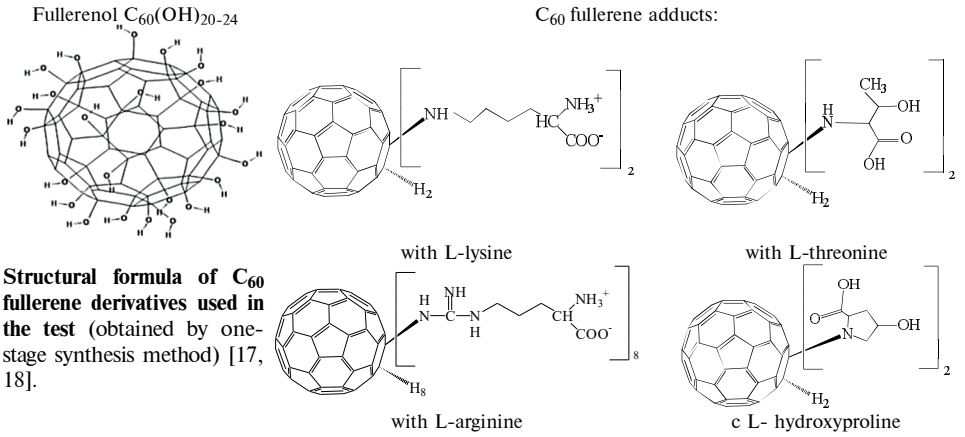
At the end of each greenhouse trial, the biometric parameters of plant growth were measured, including the total weight and biomass of leaves, roots, and stems. Assimilating leaf surface area (S) was calculated by formula:

$$S = (P \times S_1 \times n)/P_1,$$

where S_1 is the area of a cutting; n is the number of cuttings; P is the total leaf weight, g; P_1 is weight of cuttings, g.

Data processing was performed with MS Excel 2003 software and Statistica 8 (StatSoft Inc., USA). The arithmetical mean values for the parameters (M) and their confidence range (±SEM) at 95 % probability level by the Student's t-test are given. In greenhouse traits, where the sample size was small (up to 30 plants), the significance of the differences between the test variants was also determined by a nonparametric Wilcoxon test.

Results. The figure shows structural formulas of synthesized water soluble C_{60} derivatives, the fullerenol, C_{60} adducts with amino acid L-lysine, L-threonine, L-arginine and L-hydroxyproline. Amino acids for fullerene derivatives synthesis are chosen because lysine, threonine and arginine are indispensable amino acids not synthesized in humans and animals, and hydroxyproline is interesting due to a nonspecific increase in plants stress resistance



Structural formula of C_{60} fullerene derivatives used in the test (obtained by one-stage synthesis method) [17, 18].

Assessment of effects of the fullerene derivatives on spring wheat and spring barley plants indicates that the presence of these compounds in the root area promotes larger leaves area with higher chlorophyll content (Table 1). Thus, in Leningradskaya 6 spring wheat these changes were more pronounced than in Leningradskaya spring barley. The obtained data show that the functional potential of photosynthetic apparatus influenced by fullerene derivatives is higher compared to control, that leads to an increase in the net productivity. Ch. Wang et al. [16] reported about the ability of fullerenol to increase the chlorophyll synthesis in leaves of 7-day old seedlings of spring wheat.

Note, the barley plants treated with amino acid derivatives of fullerene and the corresponding amino acids do not differ significantly in leaf content of chlorophylls (ChlRI) and anthocyanins (ARI) (see Table 1), while in wheat

plants the threonine and hydroxyproline derivatives in the nutrient solution have significantly more pronounced stimulating effect on ChlRI and inhibiting effect on ARI than the amino acids themselves.

1. Physiological parameters and net productivity (deviation from control, %) **in Leningradskii spring barley and Leningradskaya 6 spring wheat in the presence of C_{60} fullerene derivatives in the root area** (greenhouse trials)

Compound	Pigment content		LA	Dry weigh			
	ChlRI	ARI		roots	stems	leaves	total
Barley of Leningradskii variety							
$C_{60}(OH)_{20-24}$	+13	–17*	+19*	+105*	+79*	+54*	+67*
C_{60}-arginine	+8	–10	+16*	+38*	+22*	+15*	+27*
Arginine	+9	–15*	+54*	+34*	+22*	+26*	+25*
C_{60}-lysine	+12	–13*	+26*	+28*	+41*	+33*	+32*
Lysine	+13	–19*	+36*	+76*	+67*	+46*	+63*
C_{60}-threonine	+16*	–25*	+15*	+69*	+69*	+32*	+50*
Threonine	+13	–18*	–9	+27*	+7	+2	+10
C_{60}-hydroxyproline	+11	–23*	+0,5	+85*	+60*	+33*	+54*
Hydroxyproline	+8	–13*	+30*	+78*	+30*	+38*	+45*
Wheat of Leningradskaya 6 variety							
$C_{60}(OH)_{20-24}$	+54*	–17*	+89*	+296*	+275*	+177*	+226*
C_{60}-arginine	+23*	–7	+20*	+83*	+174*	+112*	+124*
Arginine	+11	–9	+60*	+77*	+165*	+128*	+124*
C_{60}-lysine	+19*	–10	+60*	+80*	+133*	+107*	+110*
Lysine	+20*	–11	+73*	+96*	+170*	+133*	+134*
C_{60}-threonine	+32*	–24*	+38*	+84*	+188*	+112*	+127*
Threonine	+14*	–8	–37*	+87*	+14*	+3	+20*
C_{60}-hydroxyproline	+47*	–17*	+49*	+138*	+284*	+178*	+197*
Hydroxyproline	+8	–3	–19*	+133*	+40*	+35*	+49*

Note. The averaged data of two tests under controlled conditions (n = 30 in each) are shown. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). ChlRI and ARI are reflection indices of the content of chlorophylls (ChlRI) and anthocyanins (ARI) in plant leaves (formulas to calculate the indices were given earlier) [23, 24]; LA is assimilating leaf area. Biomass was weighed at stem elongation phase (leaf 6-7).
* Differences with control are statistically significant at $p < 0.05$.

2. Biomass accumulation in foliar treatment of plants with nanocomposition based on C_{60} fullerene derivatives (greenhouse trial)

Variant	Aerial parts		Roots	
	g, M±SEM	deviation from control, %	g, M±SEM	deviation from control, %
Barley				
Belogorskii variety				
Solution	5.50±0.26		2.73±0.30	
Solution + $C_{60}(OH)_{20-24}$	5.93±0.48	+7.8	2.97±0.31	+8.8
Leningradskii variety				
Solution	2.10±0.20		0.50±0.05	
Solution + $C_{60}(OH)_{20-24}$	1.98±0.20	–5.7	0.46±0.06	–8.0
Solution + C_{60}-threonine	2.00±0.20	–4.8	0.70±0.06*	+40.0*
Solution + threonine	1.97±0.22	–6.2	0.50±0.06	0
Wheat				
Leningradskaya 6 variety				
Solution	0.75±0.14		0.33±0.08	
Solution + $C_{60}(OH)_{20-24}$	0.76±0.18	+1.3	0.31±0.06	–6.1
Solution + C_{60}-threonine	0.79±0.16	+5.3	0.40±0.06*	+21.2*
Solution + threonine	0.75±0.16	0	0.36±0.08	+9.1

Note. In each variant n = 50. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). Concentrations of C_{60}-threonine and $C_{60}(OH)_{20-24}$ are 0.1 mg/l and 15 mg/l, respectively. Biomass was weighed at stem elongation phase (leaf 6-7).
* Differences with control are statistically significant at $p < 0.05$.

According to the available data, the induction of anthocyanin synthesis is a nonspecific response to various abiotic stressors, UV-B radiation, soil drought, and nitrogen deficit [23, 24]. It was found that the C_{60} fullerene derivatives introduction into the root area decreased the leaf level of anthocyanins in barley and wheat plants. This indirectly indicates an improvement in the physiological state of

plants, which is confirmed by higher growth parameters. Under the effect of C_{60} fullerene derivatives, the roots are extended, plants are higher and have more stems (data not given), which results in higher biomass of leaves, stems, roots and a plant as a whole. While fullerene derivatives are introduced into the root area, the total dry weight (roots + stems + leaves) for both crops overcomes controls by 27-226 % ($p < 0.05$) (see Table 1). In foliar treatment with the nanocomposition of fullerene derivatives, the dry weight of the aerial parts of barley and wheat plants did not differ significantly from the controls, while the root biomass was 8-40 % higher (Table 2).

Parametric tests and nonparametric Wilcoxon test showed the reliability of differences between the control and application of fullerene derivatives. Fullerenol, fullerene C_{60}-oxyproline, and fullerene C_{60}-threonine caused the most pronounced increase in growth indicators compared to control. The nutrient solution combined with hydroxyproline, threonine and, to a lesser extent, arginine derivatives of C_{60} fullerene have significantly higher positive effect on leaves, stems and roots compared to the same solution but containing only a corresponding amino acid. Inversely, in pairwise comparison of amino acid derivatives of fullerenes and the corresponding amino acids, the positive effect of lysine is higher compared to that of lysine adduct of C_{60}.

In our tests, all C_{60}-fullerene derivatives significantly influenced the antioxidant systems of barley and wheat plants, with more apparent effect of the hydroxyproline and threonine derivatives. However, the antioxidant response of these two cereals was somewhat similar, but somewhat different (Table 3). Intensity of LP in leaves and roots decreased by C_{60}-threonine and C_{60}-hydroxyproline, mostly reliably or as a trend. The decrease was more apparent in barley leaves (by 18 and 20 %, $p < 0.05$), in wheat leaves with C60-threonine (by 10 %, $p < 0.05$) and in wheat roots with C_{60}-hydroxyproline (by 30 %, $p < 0.05$). In barley, SOD activity did not significantly change in the leaves and increased by 17-18 % ($p < 0.05$) in the roots under the impact of both amino acid derivatives. Wheat plants manifested a weak tendency to a decrease of this index in leaves with C_{60}-hydroxyproline and C_{60}-threonine and reliable reduction of SOD activity in the roots (by 21 %, $p < 0.05$) with C_{60}-threonine.

3. Antioxidant activity of Leningradsii spring barley plants under root treatment with the C_{60}-hydroxyproline and C_{60}-threonine (greenhouse trial)

Index	Leaves			Roots		
	control value	absolute value	deviation from control, %	control value	absolute value	deviation from control, %
			C_{60}-threonine			
LP, mM/g	0.0065	0.0052*	–20*	0.0069	0.0064	–7
SOD, relative units	1.0680	1.0770	+1	1.0770	1.2635*	+17*
ROS, relative units	3.3300	6.2300*	+87*	0.3300	1.0300*	+212*
			C_{60}-hydroxyproline			
LP, mM/g	0.0065	0.0053*	–18*	0.0069	0.0069	0
SOD, relative units	1.0680	0.9513	–11	1.0770	1.2669*	+18*
ROS, relative units	3.3300	3.8700*	+16*	0.3300	0.6300*	+91*

Note. The averaged data of two tests under controlled conditions (n = 30 in each) are shown. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). LP is lipid peroxidation, SOD is superoxide dismutase, ROS is reactive oxygen species.

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

Changes of ROS content in leaves and roots under the effect of amino acid derivatives of C_{60} are the most contrasting in comparing two crops and vary from a reliable increase in barley roots (by 91 and 212 %, $p < 0.05$) and leaves (by 16 and 87 %, $p < 0.05$), and also in wheat roots (by 56 %, $p < 0.05$) with C_{60}-hydroxyproline to a decrease in wheat roots and leaves (by 61 and 71 %, $p < 0.05$) with C_{60}-threonine and a tendency to decline in the wheat leaves with C_{60}-hydroxyproline.

4. Antioxidant activity of Leningradskii spring barley and Leningradskaya 6 spring wheat under foliar treatment with C_{60}-hydroxyproline and C_{60}-threonin (greenhouse trial)

Index	Leaves			Roots		
	control value	absolute value	deviation from control, %	control value	absolute value	deviation from control, %
Barley of Leningradskii variety						
C_{60}-threonine						
LP, mM/g	0.0069	0.0049*	–29*	0.0068	0.0134*	+97*
SOD, relative units	0.7810	1.1925*	+53*	1.3396	1.1148*	–17*
ROS, relative units	5.2000	4.0000*	–23*	0.2700	1.4700*	+444*
C_{60}-hydroxyproline						
LP, mM/g	0.0069	0.0054*	–22*	0.0068	0.0064	–6
SOD, relative units	0.7810	1.2123*	+55*	1.3396	1.0985*	–18*
ROS, relative units	5.2000	5.1000	–2	0.2700	1.5000*	+456*
Wheat of Leningradskaya 6 variety						
C_{60}-threonine						
LP, mM/g	0.0060	0.0057	–5	0.0095	0.0098	+3
SOD, relative units	0.9933	1.1542*	+16*	0.8738	0.9294	+6
ROS, relative units	1.0700	1.9700*	+84*	0.9700	0.0300*	–97*
C_{60}-hydroxyproline						
LP, mM/g	0.0060	0.0046*	–23*	0.0095	0.0067*	–30*
SOD, relative units	0.9933	1.0240	+3	0.8738	0.9360	+7
ROS, relative units	1.0700	0.5000*	–53*	0.9700	0.9700	0

N o t e. The averaged data of two tests under controlled conditions (n = 30 in each) are shown. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). LP — lipid peroxidation, SOD — superoxide dismutase, ROS — reactive oxygen species.

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

In foliar treatment of barley and wheat plants with fullerene derivatives, the LP, SOD and ROS values change in a similar way when these compounds enter root area (Table 4). The antioxidant effect of foliar C_{60}-threonine and C_{60}-hydroxyproline was higher in plant leaves, i.e. predominantly, a decrease in LP and ROS content and an increase in SOD activity. In the roots, contrarily, oxidation increased, especially in barley. In barley, foliar treatment with fullerene derivatives, like root treatment, elevated sharply the root level of ROS (by 444 and 456 %, $p < 0.05$), but SOD activity and LP changed in roots oppositely, i.e. SOD significantly decreased and LP increased with C_{60}-threonine and did not change significantly with C_{60}-hydroxyproline. In wheat, foliar C_{60}-hydroxyproline resulted in a significant decrease in LP, while C_{60}-threonine decreased ROS level, with no significant differences from control for other estimated indices.

Such ambiguous results can be due to different sensitivity of the studied varieties to impacts [25]. So, in barley, more responsive to test substances, higher ROS level in roots and leaves upon root treatment, and also in roots under foliar treatment indirectly indicates possible involvement of active oxygen species in constructive plant metabolism, and also, apparently, immunomodulating effect of C_{60}-hydroxyproline and C_{60}-threonine derivatives. This effect is similar to that of a vaccine which activates an immune response to a potentially dangerous factor before its impact with a significant enhancement of plant resistance. Less reactive wheat plants, as compared to barley plants, showed more apparent direct anti oxidative effects of amino acid derivatives, both upon root and foliar treatment.

Thence, the influence of studied fullerene derivatives on growth and net productivity of plants is associated with their significant regulatory impact on synthesis of plant pigments, efficiency of photosynthetic apparatus, and also on antioxidant protection against oxidative stress.

Earlier we revealed the ability of fullerenol, a polyoxyhydroxylated fullerene derivative, to prevent oxidative stress in roots of cereal seedlings via a decline of free radical level under stressors (UV-B radiation, salinity or salicylate in the root area) and, thus, to enhance plant resistance during early phases of

growth [9].

Detected effects of fullerene derivatives on barley and wheat plants under favorable conditions gave us reason to predict an increase in plant resistance to factors that cause oxidative stress. Our experiments to simulate stress during stem elongation which is the most susceptible period of cereal plant growth, confirm protective functions of fullerene derivatives upon foliar application. By the example of Leningradskii spring barley, it was found that on day 2 after UV-B irradiation the chlorophyll content decreased significantly (by 4 %) in the leaves of control plants and did not change in the leaves of plants pretreated with C_{60}-threonine (see Tables 2, 5). The same was observed on day 7 after UV-B radiation. The leaf level of anthocyanins on day 2 after irradiation increased by 20 % in control plants, by 13 % in those treated with C_{60}-threonine and by 8 % after treatment with threonine. After 7 days, the anthocyanins in the control irradiated plants remained 20 % higher than in control (without irradiation), while this level increased by 17 % with C_{60}-threonine and by 30 % with threonine. This suggests that the impact UV-B radiation stress on plants pretreated with C_{60}-threonine is less significant compared to that of control irradiated plants and the plants pretreated with threonine.

5. Leaf pigment contents in Leningradskii barley variety under foliar treatment with amino acid derivatives of fullerene (C_{60}-threonine as the example) (greenhouse trial)

Variant	ChlRI, relative units		ARI, relative units		ChlRI, % to control		ARI, % to control	
	day 2	day 7	day 2	day 7	day 2	day 7	day 2	day 7
Solution	0.512	0.478	0.521	0.482	100	100	100	100
Solution + C_{60}-threonine	0.523	0.484	0.486	0.494	102	101	93	103
Solution + threonine	0.508	0.497	0.479	0.506	99	104	92	105
Solution + UV-B	0.492	0.456	0.627*	0.578*	96	95	120*	120*
Solution + UV-B + C_{60}-threonine	0.512	0.494	0.587*	0.565*	100	103	113*	117*
Solution + threonine + UV-B	0.507	0.471	0.563*	0.624*	99	99	108*	130*

N o t e. In each variant $n = 50$. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). C_{60}-threonine concentration is 0,1 mg/l. UV is UV-B irradiation of plants at 20 kJ/m^2; plants not treated with C_{60}-threonine and not subjected to UV-B irradiation are the control.

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

6. Spring barley plant biomass after UV-B irradiation (deviation form control, %) under foliar application of nanocomposition based on C_{60}-fullerene derivatives (greenhouse trial)

Variant	Aerial parts	Roots
	Belogorskii variety	
Solution + $C_{60}(OH)_{20-24}$	+7.8	+8.8
Solution +UV-B	–32.7*	–9.5
Solution + UV-B + $C_{60}(OH)_{20-24}$	–13.1	+11.7
	Leningradskii variety	
Solution + C_{60}-threonine	–4.8	+40.0*
Solution + threonine	–6.2	0
Solution+ UV-B	–33.3*	–20.0*
Solution+ UV-B + C_{60}-threonine	–23.8*	–2.0
Solution + threonine + UV-B	–28.6*	–8.0

N o t e. In each variant $n = 50$. Composition of aerated 20 % nutrient solution with micro- and macronutrients was described earlier [9], day/night is 14/10 h (DNT-400 lamps, 80-90 W/m^2 of photosynthetically active radiation). Concentration of C_{60}-threonine is 0.1 mg/l, of $C_{60}(OH)_{20-24}$ — 15 mg/l. Biomass was weighed at stem elongation phase (leaf 6-7). UV-B dose is 20 kJ/m^2.

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

As per weight of the aerial parts and roots, the barley plant tolerance to UV-B irradiation was significantly higher (by 10-20 %) after treatment with nanocomposition. In the control irradiated plants under impact of the stressor, the weight of the aerial parts reduced by approximately 33 %, of roots — by 10-20 % (Table 6). Note a tendency to more pronounced effect of a threonine derivative of fullerene compared to threonine.

Thus, C_{60} fullerene derivatives, the fullerenol and C_{60} fullerene adducts with threonine, lysine, arginine, hydroxyproline, stimulate growth and net productivity in spring wheat and barley under controlled conditions. This is due to the regulatory effect on synthesis of photosynthetic pigments and activity of photosynthetic apparatus, as well as to the antioxidant effect that enhances the protection of plants from oxidative stress. These changes are the most apparent under application of fullerenol, C_{60}-threonine and C_{60}-hydroxyproline. The stress resistance under UV-B radiation of barley plants treated with these compounds was 10-20 % higher than in the irradiated control plants. The obtained data indicate the necessity for further study of the impact of water soluble fullerene derivatives on plants and their habitat to create effective and environmentally friendly preparations for crop production in which high efficiency at low concentrations are combined with low costs due to a solid powder form (unlike liquid analogues).

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QTL MAPPING IN HEXAPLOID SOFT WHEAT (*Triticum aestivum* L.) IN WEST SIBERIAN PLAIN

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Abstract

Mapping of quantitative traits loci (QTL) is a modern approach to studying their genetic variability. In this, mapping QTL which determine the economically valuable traits and their effective use in the marker assisted selection are of practical interest. Here, we report evaluation of a set of 114 recombinant inbred lines of spring wheat (*Triticum aestivum* L.) mapping population ITMI (International Triticeae Mapping Initiative) in the conditions of West-Siberian plain, Russia. The ITMI mapping population was obtained by crossing spring wheat *Triticum aestivum* L. cultivar Opata 85 with a synthetic hexaploid W7984, the amphidiploid which was produced by crossing *Aegilops tauschii* Coss. (DD) sample CIGM86.940 and tetraploid wheat *T. turgidum* var. *durum* cultivar Altar 84 (AABB). In total, 42 different economically valuable traits were evaluation during the vegetation period, and 55 quantitative trait loci were identified. The dependence fidelity between the identified loci and trait polymorphism was estimated based on the threshold of the likelihood ratio of LOD-score (logarithm of odds). For 35 identified QTL, LOD ≥ 3.0 was found. Identified QTL were dispersed on 19 linkage groups different chromosomes and expressed in environment conditions of southern forest-steppe zone of West-Siberian plain with varying certainty. It was shown that the manifestation of the identified QTL may be environmentally dependent or independent, and the investigated quantitative traits correlated and were interrelated. To determine the nature of the relationship between the evaluated traits, the correlation coefficients r_{xy} were calculated. We revealed different correlations between expression of the evaluated economically valuable traits studied which stresses on the complex nature of their manifestation. It is established that the genetic variability of most of the traits evaluated is usually controlled by several QTL with broad effects which correlate with one another or by a large number of QTL with small effects. The detected QTL and linked molecular markers may be of interest for further study of the genetic control of economically valuable traits determined by identified QTL and for implementing marker-assisted selection in bread wheat.

Keywords: *Triticum aestivum*, quantitative economically valuable traits, ecology and genetic mapping, southern forest-steppe zone of West-Siberian plain of Russia

Mapping of quantitative traits loci (QTL) is a modern molecular approach to selection based on polygenic traits, including marker-assisted selection (MAS) [1-3]. The purpose of these researches is to identify, to study, to map and to introduce QTLs, effectively influencing variation of phenotypic traits, into varieties and lines which are of breeders' interest. The modern QTL mapping methodology is described in detail [4-6], including publications in Russian [7-9]. Summing up the available information, it can be concluded that in the

absence of expressed molecular genetic differences between the genes types, regulating quantitative and qualitative characteristics, gene mapping techniques using for qualitative (Mendelian) factors is not directly applicable to quantitative traits, in which case offspring individuals cannot be classified or separated into discrete phenotypic classes.

Currently, there are practical results of QTL analysis used both for mapping of identified QTLs and for their cloning and transfer to lines and varieties [8]. Estimation of environmental impact on the manifestation of studied traits is one of the most important steps in QTL mapping. Breeders can use only reproducible data of QTL analysis [10]. As QTL manifestation can depend on the environmental impact, the breeder can adjust the plant growing conditions (for example, under the Precision Farming Program) [11] to provide manifestation of the needed traits. Consequently, the QTL mapping at different ecological and geographical areas have both fundamental and practical breeding importance. The ecological genetic QTL mapping started from the papers in which it was shown that the QTL localization can vary in different experiments and relatively to other QTL, indicating a significant effect of external conditions on trait manifestation [12, 13].

To establish the genotype × environment interactions, the stability of QTL location in the identified linkage groups was tested depending on external conditions. In tomato, 350 F_2 descendants were grown in three different eco-geographic locations (two in the USA, one in Israel). As a result, the authors identified 29 QTLs; 15 were specific for only one area and only 5 were common to all three [12]. At the same time, corn cultivated in the United States at the six areas (two in the states of North Caroline, Iowa and Illinois), were weaker influenced by the environment [13]. In this experiment, the total number of the identified QTLs was higher. One hundred twenty six doubled-haploid rice lines were studied in nine geographical locations of Philippines [14]. Assessing the height of plants and the time of ear formation, the authors identified 37 main QTLs determining these traits, and 29 QTL with an epistatic effect. In Italy, QTL controlling grain protein content [15] was studied. Because this trait also depends on growing conditions, 65 recombinant inbred lines (RIL) of tetraploid wheats were used for QTL identification. As a result, authors localized seven QTLs for grain protein content (GPC) located on the chromosome arms 4BS, 5AL, 6AS (two loci), 6BS, 7AS and 7BS, and their manifestation directly depended on the growing conditions. These data are similar to obtained on hexaploid wheat by Russian scientists together with German colleagues [16, 17], when the mapping population was grown in Germany. The authors evaluated 51 RILs of soft wheat. As a result, 32 loci on 12 chromosomes of different homeological groups were mapped by 14 traits associated with the indicators of soft wheat quality. Earlier, the same German colleagues mapped QTLs determining agronomically important traits of the same RIL [18]. In contrast to the previous research, the authors used 114 RILs and made observations at three geographical locations in Germany for four vegetation periods. As a result, they mapped 210 QTLs with a minor effect and about 64 basic QTLs that determine the morphological and economically valuable traits of soft wheat [18]. However, it should be pointed out that, at the seeming wide scale of this work, the plot size in the experiment was 3, 4, 5 and 9 m^2 for each vegetation period, and the assessment for only 5 genotypes (individuals) for each RIL mapping population was performed. In addition, the eco-geographic conditions of Germany, of course, differ from Russian (at least because of the difference in geographical location of the countries).

In Russia, QTL mapping of higher plants in different eco-geographic areas has been carried out since 2005 [10]. So, a group of Russian and German scientists performed a series of studies on the mapping RIL population in various eco-

geographic zones, as well as under controlled conditions of an agroecobiological testing ground (agroecobiopolygon) [10, 19-21]. It is found that QTLs can either depend on environmental conditions, or not manifest such dependence. The reliability of the interrelation between the identified QTL and the polymorphism for one or another parmeter was evaluated based on the threshold value of the likelihood ratio of the logarithm of odds (LOD-score). In some cases, the localization of the identified QTL position in the linkage groups persisted for a number of years and in different areas, although the LOD value could vary. In addition, the QTL analysis revealed the block structure of the *T. aestivum* genome and the percentage of phenotypic variability determined by each of the identified QTLs, and also which of the parents introduced the QTL allele.

Important results are obtained by estimation of the number and exact chromosomal localization of the QTLs involved in the physiological and genetic control of complex agronomically important traits of spring wheat *(T. aestivum*) under the conditions of agroecobiopolygon [21] where it is possible to selectively modify an analyzed environmental parameter, keeping all the others without changes. These results unambiguously confirmed that in fixed conditions the QTL localization is stable, and the manifestation of some QTLs is associated with the impact of certain external factors. One-factor analysis of variance showed that the change in the temperature and light regime did not affect only 21 of 30 estimated traits (70 %). Four of the remaining traits that are susceptible for temperature and light impact, are directly associated with grain efficiency, which determines not only their economic value, but also the importance for the survival, preservation and species distribution. Four more traits perform a protective adaptive function in vegetation, and one is realized during initial growth and development, participating in the initiation of a cascade of physiological and genetic mechanisms that ensure the maximum possible performance of plants in specific environmental conditions.

In spite of considerable number of papers on genetic and environmental QTL mapping in Russia, information about the genome functioning and mechanisms of manifestation of commercially important traits of spring wheat is still not complete. In the present research, we for the first performed a large scale test of 114 recombinant inbred lines of the ITMI (International Triticeae Mapping Initiative) of hexaploid spring wheat in the conditions of Omsk city. As a result, QTLs for 42 agronomically important traits are identified and localized. The QTL locations depend on the eco-geographical conditions of plant growing. It is shown that the studied quantitative traits are interrelated. Molecular markers linked to detected and identified QTL are established.

The aim of the research was to identify and to map loci that determine economically valuable traits of soft wheat in the conditions of southern forest-steppe areas of the West Siberian Plain.

Techniques. The recombinant inbred lines (RIL) of hexaploid spring wheat (*Triticum aestivum* L.) of mapping population ITMI (International Triticeae Mapping Initiative) were used. The ITMI population was created by pollination of spring Opata 85 soft wheat (*Triticum aestivum*) variety with the pollen of the synthetic hexaploid W7984. Amphidiploid W7984 was obtained by crossing *Aegilops tauschii* Coss. (CIGM86.940, DD) as a male parent and tetraploid wheat *T. turgidum* var. *durum* Altar 84 (AABB) variety as a female parent [19, 20]. In these papers [19, 20] we also described selection of genotypes for the RILs of ITMI mapping population and their properties.

RILs of ITMI were grown and evaluated on an experimental field in 2016 in the conditions of the southern forest-steppe of the West Siberian Plain, in the center of the southern part of the Omsk area. The Omsk area is a zone of insuffi-

cient moisture. The limiting factors in the region are periodically recurring droughts of different intensity and epiphytotics of brown and stem rust [22]. In general, the weather conditions of the vegetation period of 2016 were quite favorable and typical for the region: in the first half of June, there were high temperatures and no precipitation, in July the warm, rainy weather favorable for plant development was prevalent, and in August the weather was warm and dry. On some days the maximum air temperature in June, July and August reached 32-35 °C. Brown and stem rust infections were noted in the third decade of August.

Manual sowing of recombinant inbred lines of the ITMI mapping population (40 grains per a row at 5 cm depth, 1 m wide strips with 15 cm row spacing) was performed on May 17, 2016. Manual harvesting (each row separately) was carried out on September 5.

The analysis of trait was performed according to the VIR methodology [23]. The traits with expressiveness sufficient for estimation were scored. A total of 42 traits were analyzed during the growing season.

QTL analysis was performed with MAPMAKER/QTL program [18, 24]. As this program in based on the J.B.S. Haldane's formula [25], MAPMAKER/EXP 3.0 software [24] and the data of the GrainGenes database (gopher: http://www.greengenes.cit.cornell.edu) were used for re-calculation of distances. The results of phenotype analysis were integrated into the existing basic map of ITMI population [26]. QTL localization in the linkage groups was identified with QGENE [27] as described [18-20], using only the markers which correspond to the D.D. Kosambi's mapping function considering interference [28].

The reliability of interrelation between the identified loci and polymorphism on a trait was evaluated based on the threshold value of the likelihood logarithm of odds (LOD-score ratio) [29, 30]. For each trait in each experiment, individual QTL analysis was performed and the degree of variations (R^2) was determined for phenotypic traits associated with the corresponding QTL. Significance of each LOD was determined by permutation test (1000 replications). Only loci with LOD $\geq$ 3.0 ($p < 0.001$), 2 < LOD < 3 ($p < 0.01$) and 1.5 < LOD < 2 ($p < 0.1$) [18-20] were considered.

Correlation coefficients r_{xv} were calculated to determine the nature of the interaction contingency between the traits. Under the assumption that (x_1, y_1), (x_2, y_2), ..., (x_n, y_n) is a sample of n observations for a pair of variables (x, y), the correlation coefficient r_{xv} is calculated as follows [31]:

$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2 \sum_{j=1}^{n}(y_j-\bar{y})^2}},$$

where $\bar{x}$, $\bar{y}$ are selective averages calculated as

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i\,,\ \bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i.$$

Negative correlations indicate the relationship between the traits, at which the mean values of one trait decrease with increasing values of the other trait.

Correlation coefficients were reliable at $p < 0.05$. Such a value is an acceptable boundary of statistical significance, because it includes an error rate of 5 % [32]. All indicators were calculated using the STATISTICA 10.0 software (StatSoft, Inc., USA).

Results. Molecular mapping (Table 1 at the http://www.agrobiology.ru) showed that sprouting-tillering period is determined by a QTL located on chromosome 6B. The phenotypic variation associated with the allele obtained from the male W7984 parent was rather high and reached 18.70 %. The sprouting-booting period was also determined by one QTL which was located on chromosome 5A (phenotypic variability was 5.60 %), but in this case the allele was in-

troduced by the female Opata 85 form.

QTLs for sprouting-earing and sprouting-maturation periods, as well as for plant shape were located on chromosome 5D. All of the identified QTLs were located close to each other, but the percentage of phenotypic variation for each QTL for these three traits varied (from 10.05 to 18.97 %). One of the two QTLs associated with plant height was also mapped on chromosome 5D. The second QTL of the same trait was on chromosome 4A. Note, all the QTLs identified in the 5D linkage group are introduced by the male parent, and the allele on chromosome 4A is inherited from the female one.

Three QTLs (linkage groups 1A, 3A and 7A) determine the length of the upper internode, alleles on 1A and 3A are obtained from the female parent, on 7A from the male parent. The observed phenotypic variation was quite high and varied from 23.48 % (7A) to 28.69 % (1A, 3A). A single QTL on 3D determines the flag position at the beginning of earing, with lower percentage of phenotypic variation (only 14.03 %), although the LOD score is 3.25.

The location of QTLs identified on chromosomes 2D and 7D and determining the wax appearance on stem and front side of leaf coincides completely, although phenotypic variation ranges from 28.52 % (7D) to 52.42 % (2D). The wax coating on the ear is also determined by two QTLs, on 2D and 1D (27.32 and 12.77 % phenotypic variations, respectively).

QTLs for traits determining yield (ear length, spikelet number per ear, grain number per spikelet, grain number per ear, grain weigh per ear, 1000 grain weigh and ear number) are identified in seven different linkage groups. In this case, QTLs for grain number per spikelet, grain number per ear, grain weight per ear are located in one site on chromosome 1B, for 1000 grain weigh and ear number — on chromosomes 6A and 1A, respectively, and for ear length and spikelet number per ear — in the linkage groups 4A, 5D and 5A, 3A. The phenotypic variations range from 10.41 % for 1000 grain weight up to 25.07 % for ear length. Both QTL alleles associated with spikelet number per ear and also QTL allele for ear length identified on 4A chromosome are introduced from the Opata 85 variety, the rest are from W7984.

QTLs mapped on chromosomes 7B, 3B and 2B are associated with resistance to powdery mildew, brown rust, septoria and root rot, QTLs for resistance to stem rust are located on chromosomes 7A and 4B. Phenotypic variations are from 10.68 % (resistance to root rot) up to 26.75 % (resistance to stem rust). The QTL alleles for resistance identified on 7B, 4B and 2B are inherited from the female parent, on 7A and 3B — from the male parent.

QTLs for flag appearance, main stem leaf number and stem node pubescence are identified on chromosomes 4D, 1B and 7B, respectively. Alleles on 4D and 1B from the male parent determine 11.63 and 12.29 % of the phenotypic variations, respectively, on 7B from the female parent — 19.17 % of stem node pubescence.

Sheaf weigh with roots, plant number per sheaf, quality retained till harvesting, and grain weight per sheaf are determined by QTLs introduced only by the female Opata 85 form. QTLs for weigh with roots, plant number per sheaf are identified on chromosome 4A at 87.5 cM. QTLs for plant number per sheaf and quality at harvesting are on chromosome 5B at 128.8 cM. Identification of such QTLs and their location indicate that sheaf weigh with roots and grain weight per sheaf are determined by one locus of chromosome 4A, and plant number per sheaf and quality at harvesting are determined by one locus of chromosome 5B.

It is of interest that plant weight is determined by a locus introduced by the female form, but this QTL is mapped on chromosome 3A at 120.9 cM. Total

stem number and productive stem number are determined by QTLs identified by on 3B and 2A, respectively, and three QTLs located on chromosomes 1A, 3A and 4A at 205.7 cM, 56.3 cM and 63.2 cM, respectively, are for stem length (all these QTLs are also obtained from the female Opata 85 form).

QTL identified on chromosome 1B at 23.8 cm mainly influences the traits determining the grain yield (main ear weight, ear density, grain number per plant, grain weight per plant, 1000 grain weight per the main ear and the main ear weight). The exception is ear density and 1000 grain weight per the main ear: QTLs for these traits are identified in the linkage groups 5A and 6A, respectively. All QTLs are introduced by the male form; exception is an allele from the female form which determines ear density. The observed phenotypic variability is relatively low and alter from 10.24 % (1000 grain weight per the main ear) up to 16.84 % (grain weight per the main ear).

The coefficients of plant economic value ($K_{econ.plant}$) and the economic value of the ear ($K_{econ.ear}$) are the indices of plant and ear productivity. In Western Siberia, these values usually vary from 25 to 45 %. $K_{econ.plant}$ is calculated as the percentage ratio of the grain weight per plant to total plant weight (without roots, but with ears and grain). $K_{econ.ear}$ is the grain weight per ear percentage to total grain weight per ear. QTLs for these two traits are detected on chromosomes 1B (for both coefficients) and 5D (for $K_{econ.plant}$). All these QTLs are introduced by the male form; the phenotypic variability ranges from 11.30 % ($K_{econ.ear}$) up to 15.03 % ($K_{econ.plant}$). Their phenotypic variation is determined by the same QTL allele located on chromosome 1B at 23.8 cM.

Note, a locus identified on chromosome 1B at 23.8 cM includes genes which are responsible for plant productivity and grain yield traits (grains number per spikelet, grain number per ear, grain weight per ear, the main ear weight, grain number per plant, grain weight per plant, grain weight per the main ear, $K_{econ.plant}$ and $K_{econ.ear}$). QTL determining leaf number per the main stem is also located on chromosome 1B, but at 176.0 cM, so it is another locus different from the 23.8 cM locus. The 1000 grain weight and 1000 grain weight per the main ear are also determined by one locus mapped on 6A at 101.9 cM. Two blocks of genes are identified on chromosome 4A, which are formed by loci at 87.5 cM (for sheaf weight with roots and grain weight per sheaf) with a nearby loci at 63.2 cM (for stem length) and 206.5 cM (for ear length and plant height). The presence of such gene blocks assembled in one or more linkage groups indicate an evolutionary co-inheritance of the traits which are responsible for certain stages of plant ontogenisis and its individual features under specific growth conditions. Of course, canalization of variability and conservatism of genetic systems of ontogenetic and phylogenetic adaptation (F and R systems), as well as their subcomponents are rather relative in natural evolution, but are significant for human practical activities, especially in breeding [33]. Thence induced recombinogenesis plays the special role in increasing efficiency of breeding programs [34]. Genomic maps facilitate dividing quantitative trait into simpler genetic components (QTLs) and simplify their identification, especially under genotype × environment interaction. Obviously, the character of this interaction depends on plant adaptive potential [34-38]. Effects of environmental factors apparently much determine the evolutionally formed blocks of co-adapted genes in each species, including wheat, and also the co-adaptation specificity of its genetic system as a whole. This is the base for evolutionary and ontogenetic "memory" of the genetic F and R systems which are specific to each plant species [33, 34, 38-40]. Theoretically, QTL can be detected only if the parents carry different alleles [4, 9]. The desired allele may be very specific for one parent and absent in other genotypes, for example, in those making up the mapping popula-

tion. Nevertheless, the identified QTLs indicate the possibility to improve breeding material by grouping chromosome regions with the desired positive effects. Our research revealed that the quantitative traits correlate with each other. This follows from the presence of more than one QTL for two or more traits in the same locus on the chromosome and the nature of their manifestation. However, the obtained results do not allow us to separate the effects of close loci linkage and pleiotropy. To establish statistically reliable interactions of the studied traits, we performed correlation analysis (Table 2, http://www.agrobiology.ru).

The correlation analysis identified completely linear relationship (with r almost equal to 1) between plant height and ear length, and plant number per sheaf and quality at harvesting (here and farther, only the results that are statistically significant at $p < 0.05$ are considered). Thence the correlations of these pairs with the rest traits coincide.

The correlations of sprouting—tillering period with sprouting—booting ($r = 0.73$) and sprouting—maturation ($r = 0.79$) periods are rather high. Waxy coating of the outer side of leaves tightly correlates with the wax appearance on stem ($r = 0.98$) and ear ($r = 0.77$). A strong correlation ($r = 0.75$) is also found between waxy coating on stem and on ear.

Grain numbers per ear and per spikelet correlate at $r = 0.91$. These traits also have high correlation coefficients with grain weight per ear (0.89 and 0.84, respectively), with the main ear weight (0.94 and 0.86), with grain weight per plant (0.85 and 0.78), with grain number per plant (0.93 and 0.83), with $K_{econ.plant}$ (0.71 and 0.83), and with $K_{econ.ear}$ (0.78 and 0.87). Additionally, grain numbers per ear is highly associated with grain weight per sheaf ($r = 0.75$) and the main ear weight ($r = 0.73$). Spikelet number per ear also has strong correlations with plant height and ear length ($r = 0.70$). Ear number strongly correlates with sheaf weight with roots ($r = 0.79$), plant number per sheaf and quality at harvesting ($r = 0.92$).

Grain weight per ear closely correlates with grain weight per the main ear ($r = 0.97$). Estimates for the relationship between these and other tested traits are similar: $r = 0.80$ and $r = 0.82$, respectively, for grain weight per sheaf, $r = 0.86$ and $r = 0.90$ for the main ear weight, $r = 0.89$ and $r = 0.92$ for grain number per plant, $r = 0{,}92$ and $r = 0{,}94$ for grain weight per plant, $r = 0{,}73$ and $r = 0{,}74$ for $K_{econ.plant}$ and, finally, $r = 0.77$ and $r = 0.79$ for $K_{econ.ear}$. Also note the correlation coefficient between grain weight per the main ear and plant weight ($r = 0.70$).

Grain weigh per plant also shows strong correlations with grain weight per sheaf ($r = 0.87$), grain number per plant ($r = 0.96$), $K_{econ.ear}$ ($r = 0.76$), plant weight ($r = 0.78$), and the main ear weight ($r = 0.84$). In turn, the main ear weight quite strongly correlates with grain weight per sheaf ($r = 0.71$), plant weight ($r = 0.80$), grain number per plant ($r = 0.81$), and the stem length, plant height and ear length ($r = 0.72$). Correlation between stem length and upper internode length is characterized by $r = 0.86$.

It is a peculiar that grain number per plant also strongly correlates with plant weight ($r = 0.73$), grain weigh per sheaf ($r = 0.86$), and $K_{econ.ear}$ ($r = 0.77$). Note, sheaf weigh (with roots) strongly correlates with grain weight per sheaf ($r = 0.80$), plant number per sheaf and quality at harvesting ($r = 0.74$). $K_{econ.plant}$ correlates with $K_{econ.ear}$ at $r = 0.91$, total stem number and productive stem number correlate at $r = 0.89$, and correlation between 1000 grain weigh and 1000 grain weight per the main ear is at $r = 0.90$ level.

Also, we revealed medium ($0.3 \le r < 0.7$) and weak ($r < 0.3$) reliable correlations. Notably, negative correlations were also found. Sprouting—tillering period negatively correlates with sheaf weight (with roots) ($r = -0.37$), and with

ear number, plants number per sheaf and quality at harvesting ($r = -0.32$).

In general, the correlation analysis performed in this work shows that the manifestations of the studied economically valuable traits correlate, but with different strengths, which indicates their complex nature. Elucidation of genetic and physiological mechanisms underlying these traits may be the next step in practical application of identified QTLs for spring soft wheat breeding. It should be noted that this requires a complex study with a detailed planning of experiments. Nevertheless, the obtained results already make it possible to use the identified molecular markers associated with mapped QTLs in MAS.

Gene effects in different loci are commonly described by a negative exponential distribution [41]. Segregation analysis is applicable for most genes, but many genes and QTLs with small effects cannot be studied by the same method. Therefore, the hypothesis of negative exponential distribution of gene effects is not yet possible to prove or disprove, and its verification requires further research.

So, we for the first time identified and mapped QTLs for 42 economically and agronomically valuable traits of soft wheat in the conditions of southern forest-steppe of the West Siberian Plain. It is established that the genetic variability of most studied traits is usually controlled either by few correlating QTLs with wide range of effects, or by a large number of loci having small effects. The data obtained in this study allow evaluation of polygenic QTL effects and genotype × environment interaction for use in spring wheat breeding.

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GRAIN PRODUCTION AND OPTICAL CHARACTERISTICS IN THREE WHEAT (*Triticum aestivum* L.) VARIETIES UNDER LIMING AND NITROGEN FERTILIZATION

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Abstract

Liming of acidic soils occupying 73 million hectares of agricultural land in the Russian Federation is a traditional technique that provides optimization of soil conditions and contributes to obtaining high and stable yields. The cultivation of crops with intensive technologies is accompanied by the export of calcium with a yield, washing out with atmospheric precipitation, etc., so it is necessary to replenish calcium, which is economically costly. Knowing the optimum dosages for each type of ameliorant is necessary to adjust its quantity which provides the maximum effect from the application. For the first time, effect of various doses of ameliorant (limestone meal-dolomite) was evaluated in situ by the optical characteristics of spring wheat (*Triticun aestivum* L.) cultivars Leningradskaya 97, Krasnoufimskaya 100 and Trizo differing in grain productivity and responsiveness to nitrogen fertilizer application. Plants were grown in 5 litre containers with sod-podzolic soil, in natural light. The rates of applied ameliorant were 0 (control), 0.25, 0.50, 0.75 and 1.00 Hy (mmolc 100 g^{-1}). Nitrogen (ammonium nitrate) was applied before seeding in two doses: optimal (1 g N) and deficit (0.15 g N) nutrition level in 5 kg of soil. The content of other mineral nutrients in the soil was similar in all cultivars of the experiment. Spectral characteristics of radiation reflected from leave surface (300-1000 nanometers) were registered by a spectrometer HR2000 («Ocean Optics», USA). After reflection spectra recording, the spectral reflection indexes closely related to chlorophyll content, the ratio between the amount of carotenoids and that of chlorophylls, the light dispersion caused by changes of inner leaf structure, the activity of the photochemical processes of photosynthesis, anthocyanins and flavonols content were calculated. The results indicate that wheat cultivars respond differently to ameliorant and its favourable effect on plants productivity is more expressed under nitrogen deficiency. In response to ameliorant application at the optimum level of nitrogen supply, grain yield of cv. Leningradskaya 97 has not changed. Grain production of cv. Triso was higher throughout the range of application doses. Range of a positive response to the impact of ameliorant of cv. Krasnoufimskaya 100 was narrower in comparison to cv. Trizo and shifted toward lower doses. Close correlation between the grain yield under different ameliorant doses and the content of chlorophyll in the leaves (chlorophyll index) for the Krasnoufimskaya 100 ($R^2 = 0.87$) and Trizo ($R^2 = 0.88$) was found. Changes in the indices which characterize efficiency of light energy conversion in the photochemical processes of photosynthesis, allows us to suggest that the ameliorant introduction not only promotes nitrogen absorption but also affects the efficiency of light energy use through photosynthesis.

Keywords: ameliorant, wheat, grain production, nitrogen fertilizer, optical and morpho-physiological properties

In Russia, acid soils occupy 73 million hectares of agricultural lands [1], and liming is conventional method to optimize soil conditions for obtaining high and stable yields [2-5]. To date, in the Russian Federation area of ameliorative lands has reduced from 6 million to 266 thousand hectares [6]. On agricultural soils with high acidity, about 20 million tons of grain products are not annually harvested, and the payback of nitrogen fertilizers on strongly acidic soils is 1.4-2.7

times lower than on slightly acid and neutral soils [3, 6]. Intensive crop cultivation is accompanied by removing calcium with yield. According to summarized data, the annual calcium removal from soil by different species varies from 20 to 500 kg/ha [7]. In soils of the Non-Chernozem zone, atmospheric precipitations affect negatively the calcium balance. According to long-term research data, the average annual calcium losses from sod-podzol soils because of rains are 300-400 kg/ha [8]. Appreciation ща amelioration leads to the fact that the amount of introduced lime with calcium agents compensates only 6-8 % of its natural losses. Complete rejection of liming caused passing a significant part of neutral soils to the slightly acid category and slightly acid to the medium and strongly acidic categories [9].

Different response of wheat varieties to liming and their genetic heterogeneity are available resources that can ensure a reduction in crop losses caused by the soil acidity. It is shown that some wheat varieties can tolerate more acidic soil, while others are very sensitive to such conditions [10-12]. Screening for tolerance to soil acidity of 116 wheat genotypes, including commercial varieties and breeding lines from Western Australia, identified some cultivars which significantly exceeded well-known cultivated varieties in this trait [12]. Improving the situation by the genetic plant improvement for tolerance to soil acidity can be considered not only alternative to the lime or other soil-ameliorative methods, but as an auxiliary tool. Identification of responses to liming in a particular variety is necessary for the most effective ameliorant application [13]. Such approach will significantly reduce the costs for acid soil liming.

It is generally accepted that the phenotyping of economically valuable traits, such as productivity and tolerance to the abiotic stressors, is the most laborious and technically difficult because of testing in different environmental conditions for several seasons. Some of the available methods are based on the destructive sampling during different stages of plant growth, take much time and resources. In this context, new phenotyping methods with high throughput have been developed in the last decades, e.g. non-invasive imaging, spectroscopy, image analysis and high-performance computing [14, 15].

Earlier, using contact and proximity diagnostics of plants physiological state in situ, we tested a number of optical criteria (reflection indexes) for assessment of barley and wheat tolerance to different stressors, including UV radiation, water and nitrogen shortage [16-18], and also for quantitative estimates of plant requirements in nitrogen fertilizers [19]. The obtained results showed that this method reveals disorders in plants at the earliest growth stages and can be used for improving agrotechnologies and yield forecasting.

In this paper, the influence of different doses of ameliorant (dolomite flour) is evaluated for the first time in situ by optical characteristics of leaves for wheat varieties which differ in productivity and response to the nitrogen fertilizers. The optimal dosage for each variety provides the maximum effectiveness of the ameliorant application.

The aim of the paper was to find out optical criteria suitable to assess the response of spring wheat varieties grown in the northwest of the Russian Non-Chernozem zone under liming, for studying effect of the ameliorant in a wide range of doses and identification of its optimal dose for each variety depending on nitrogen nutrition.

Techniques. In the tests we used three spring wheat *(Triticum aestivum* L.) varieties, Krasnoufimskaya 100, Leningradskaya 97 and Trizo, which differ in productivity and a response to nitrogen fertilizers. The substrate was acidic (pH 4.1) sod-podzolic light loamy soil with high exchangeable aluminum and low humus content (1.9 %), hydrolytic acidity (H_h) of 4.7 mmol(eq)/100 g, Ca^{2+} of 1.75 and Al^{3+} of 0.6 mmol/100 g; the particles < 0.01 mm in size made 24.7 %.

Plants were grown in 2012 under natural light in a greenhouse containers (5 kg of soil in each) covered with plastic film. In 2012, the average monthly temperature exceeded the norm by 1.4 and 0.7 °C in May and July, and was 0.4 °C lower in August. Watering was carried out daily, soil moisture was maintained at 75±5 % of the total available soil water (TAW). Experimental design included 21 variants which differed in soil nitrogen content and hydrolytic acidity. To create different levels of nitrogen, ammonium nitrate was applied before seeding in two dosages of nitrogen, i.e. 1 g for optimal nitrogen nutrition (ONN) and 0.15 g for deficit nitrogen nutrition (DNN). The ameliorant (dolomite flour with 85 % neutralizing capacity) doses were 0 (control), 0.25; 0.50; 0.75 and 1.0 H_h. At ONN, all three verities were tested in all four liming regimes. At DNN, 0 (control), 0.50 and 1.0 H_h were tested in Krasnoufimskaya 100 and Trizo varieties, which according to preliminary data are more responsive to the ameliorant compared to Leningradskaya 97 variety. Twenty seeds were sown into each container. After emergence of seedlings, the number of plants was leveled to 12 plants per container. Plant productivity was assessed after reaching full ripening. Experiment was arranged in 4 replications.

The reflection spectra of leaf surface were registered at stem elongation and beginning of tillering in the middle parts of leaves 4 and 6 using fiber optic spectrophotometric system (Ocean Optics, USA). At least 18 spectra for each variant were recorded. The reflected radiation spectra were used to measure the light scattering by leaves R_{800} [6] and to calculate the reflection indices (chlorophyll content ChlRI, ratio of carotenoids to chlorophyll SIPI, photochemical activity of the photosynthetic apparatus PRI, content of anthocyanins ARI and flavonols FRI) characterizing the activity of the photosynthetic apparatus:

$$\text{ChlRI} = (R_{750} - R_{705})/(R_{750} + R_{705} - 2R_{445}) \text{ [20]},$$
$$\text{SIPI} = (R_{800} - R_{445})/(R_{800} - R_{680}) \text{ [21]},$$
$$\text{PRI} = (R_{570} - R_{531})\ (R_{570} + R_{531}) \text{ [21]},$$
$$\text{ARI} = R_{750}\,(1/R_{550} - 1/R_{700}) \text{ [22]},$$
$$\text{FRI} = [(1/R_{410}) - (1/R_{460})] \times R_{800} \text{ [23]},$$

where R is reflection index, figures are the wavelength reflected from the leaf surface.

For ease of the data interpretation and obtaining of positive values of reflection indexes, for all test variants, a constant C value was introduced into the PRI, ARI and FRI formulas, from which the values of these indices were deducted. The modified reflection indices are $\text{PRI}_{\text{mod}} = C_1 - \text{PRI}$, $\text{ARI}_{\text{mod}} = C_2 - \text{ARI}$, $\text{FRI}_{\text{mod}} = C_3 - \text{FRI}$. The value of C_1-C_3 was defined experimentally. Usually, C_1 value for wheat plants grown under natural light was 0.5; C_2 and C_3 were 0.7 [16, 17].

The statistical processing was performed with Statistica 8 (StatSoft, Inc., USA) software and Excel 2010. Mean values (*M*), standard errors of mean (±SEM) and confidence range at 95 % confidence level ($t_{0,05} \times$ SEM) were determined. Significance of differences between the variants was assessed by the parametric (Student's t-test) and non-parametric (the Wilcoxon pair comparison test) statistics methods. Differences between variants were statistically significant at $p \leq 0.05$.

Results. The applied optical indices make it possible to estimate the capacity (intensity) of the photosynthetic system (ChlRI is the chlorophyll reflection index which is determined by the leaf content of chlorophyll and most closely correlates with the content of chlorophyll per unit of leaf area), and the efficiency of light energy conversion in photochemical processes of photosynthesis (SIPI is the ratio of total carotenoids to total chlorophylls, PRI is the efficiency of photosyn-

thetically active radiation, ARI is the content of anthocyanins; R_{800} is criterion of light scattering which depends on the surface characteristics and leaf structure).

Plants of Leningradskaya 97, Krasnoufimskaya 100, and Trizo varieties responded differently to the ameliorant (Fig. 1). The increased grain yield (12±3 %, $p \le 0.039$) was identified in Leningradskaya 97 variety only at H_h 0.75

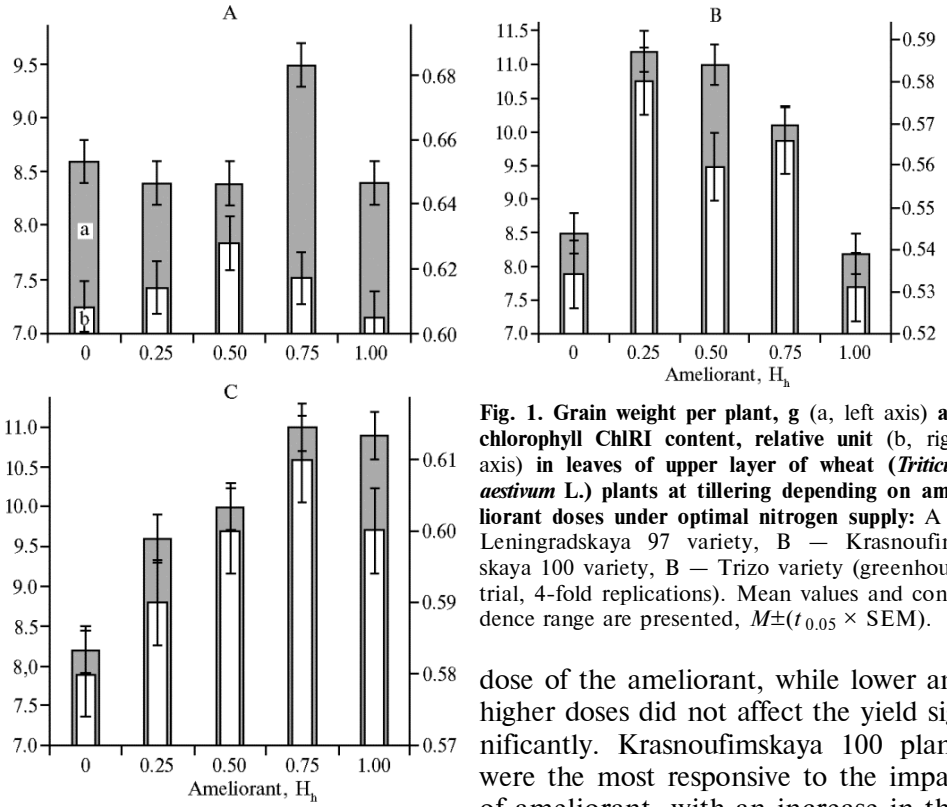

Fig. 1. Grain weight per plant, g (a, left axis) **and chlorophyll ChlRI content, relative unit** (b, right axis) **in leaves of upper layer of wheat (*Triticum aestivum* L.) plants at tillering depending on ameliorant doses under optimal nitrogen supply:** A — Leningradskaya 97 variety, B — Krasnoufimskaya 100 variety, B — Trizo variety (greenhouse trial, 4-fold replications). Mean values and confidence range are presented, $M \pm (t_{0.05} \times \text{SEM})$.

dose of the ameliorant, while lower and higher doses did not affect the yield significantly. Krasnoufimskaya 100 plants were the most responsive to the impact of ameliorant, with an increase in the productivity by more than 30±3 % ($p \le 0.024$) after the lowest dose of the ameliorant (0.25 H_h). A dose of 0.50 H_h also increased the yield of this variety by 30±2 % ($p \le 0.009$). A higher dosage (1.00 H_h) had a significant but lower effect, and the grain yield became higher by 20±3 % ($p \le 0.041$). The maximum yield increase for Trizo variety, equal to about 30 %, occurred after application of the ameliorant at 0.75 and 1.00 H_h. This variety responded positively to significantly lower doses (0.25 and 0.50 H_h), increasing the grain yield by 17±2.4 % ($p \le 0.033$) and 24±2.1 % ($p \le 0.021$), respectively. Thus, Leningradskaya 97 variety plants respond positively to relatively high dose of the ameliorant (0.75 H_h) while other doses are ineffective. Grain yield of Krasnoufimskaya 100 variety increases at all applied doses, but most significantly at 0.25 H_h whereas productivity of Trizo variety increases as the ameliorant dose elevates. It can be assumed that such a different response to the ameliorant is due to origin of varieties and properties of soils on which the plants were grown during breeding.

Changes in the wheat plants productivity depending on the ameliorant dose and nitrogen nutrition are shown in Figure 2. At ONP and 0.50 H_h ameliorant the grain weight per plant of Krasnoufimskaya 100 and Trizo varieties increased by 30 and 22 % ($p \le 0.040$), respectively. At the same nitrogen level, an increase of the ameliorant dose to 1.00 H_h did not lead higher productivity of Krasnoufimskaya 100 variety, but increased grain weight in Trizo variety by 35 % ($p \le 0.035$). In DNS and medium dose of the ameliorant (0.50 H_h), the grain weight increased by 15 % ($p \le 0.041$) in Krasnoufimskaya 100 and did not

change in Trizo plants. Grain yields of Krasnoufimskaya 100 and Trizo varieties with the 1.00 H_h ameliorant were respectively higher by 8±2 % ($p \le 0.032$) and 25±3 % ($p \le 0.021$) compared to control (ONN) (see Fig. 2). It can be concluded that in the case of a nitrogen nutrition deficiency, the ameliorant application will improve nitrogen assimilation, and to maximize the effect the ameliorant dose for Krasnoufimskaya 100 should be significantly less than for Trizo plants. Regardless of the nitrogen nutrition level, Krasnoufimskaya 100 plants are capable of the greatest yield increase with the ameliorant at 0.50 H_h. Further elevation of the ameliorant dosage does not provide an increase in grain productivity. In Trizo variety, irrespectively of the nitrogen supply, both the highest doses of the ameliorant (1.00 and 0.50 H_h) promote a significant yield increase.

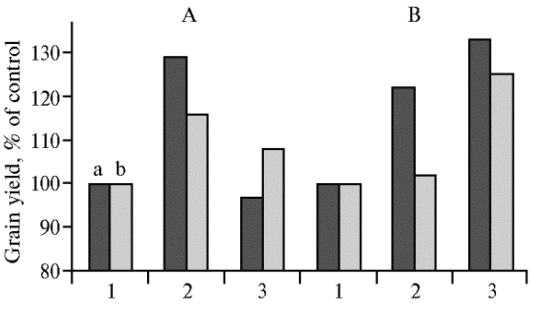

Fig. 2. **Grain weight in Krasnoufimskaya 100** (A) **and Trizo** (B) **wheat *(Triticum aestivum* L.) plant varieties as influenced by the ameliorant and nitrogen nutrition:** a — optimal nitrogen nutrition (ONN), b — deficient nitrogen nutrition; 1 (control) — without ameliorant, 2 and 3 — the ameliorant at 0.50 and 1.00 H_h, respectively (greenhouse trials, 4-fold replications). The average values are given, the grain yield without ameliorant (1) is 100 %. Confidence intervals $M \pm (t_{0.05} \times \text{SEM})$ do not exceed ±5 % ($p \le 0.05$).

The ameliorant stimulates chlorophyll synthesis (see Fig. 1) and an increase in assimilating leaf area and the percentage of leaf biomass from total plant biomass (Table 1), thus providing more active photosynthetic apparatus. Irrespectively of nitrogen supply, soil modification promotes to a significant increase in chlorophyll content. At ONP, the maximum chlorophyll increase in Krasnoufimskaya 100 variety was with 0.50 H_h of the ameliorant. Further elevation of the dose (up to 1.0 H_h) did not increase this pigment content. The Trizo variety responded positively to the ameliorant throughout the doses used: the amount of chlorophyll increased as the ameliorant dose increased regardless of nitrogen nutrition level (see Fig. 1).

1. Morphophysiological indices of wheat (*Triticum aestivum* L.) plants at earing as influenced by the ameliorant and nitrogen nutrition (greenhouse trials, 4-fold replications)

Dose, H_h	N, g per pot	ALA		Parts/plant					
				leaves		stems		ears	
		cm^2	%	g	%	g	%	g	%
Krasnoufimskaya 100 variety									
0 (control)	1	82.8	100.0	0.25	100.0	0.55	100.0	0.19	100.0
0.25	1	111.8	135.1	0.30	119.8	0.42	76.7	0.18	98.3
0.50	1	103.1	124.5	0.28	111.9	0.50	92.1	0.14	72.7
0.75	1	84.9	102.6	0.28	112.1	0.49	90.1	0.14	72.7
1.00	1	87.0	105.1	0.32	127.6	0.42	76.5	0.06	34.4
0 (control)	0.15	76.0	100.0	0.22	100.0	0.47	100.0	0.18	100.0
0.50	0.15	91.4	120.3	0.28	126.5	0.45	95.4	0.13	75.6
1.00	0.15	72.9	96.0	0.27	120.9	0.45	94.8	0.13	72.5
Trizo variety									
0 (control)	1	92.6	100.0	0.29	100.0	0.39	100.0	0.16	100.0
0.25	1	107.1	115.6	0.35	117.9	0.47	118.9	0.14	87.3
0.50	1	108.5	117.2	0.35	118.1	0.39	100.1	0.10	63.6
0.75	1	111.8	120.8	0.37	127.8	0.39	100.6	0.11	64.8
1.00	1	118.5	128.0	0.36	123.3	0.40	100.7	0.13	77.2
0 (control)	0.15	93.5	100.0	0.30	100.0	0.47	100.0	0.17	100.0
0.50	0.15	106.4	113.8	0.31	104.3	0.45	105.7	0.13	84.7
1.00	0.15	115.9	124.0	0.32	105.1	0.39	90.7	0.12	77.3

Note. ALA — assimilating leaf area. For ALA and biomass, a percentage for the control variant is indicated (for each of the varieties and for each of the doses of nitrogen) in which the ameliorant was not applied. Confidence intervals $M \pm (t_{0.05} \times \text{SEM})$ do not exceed ±5 % ($p \le 0.05$).

Determination coefficient (R^2) for the relationship between grain weight and the chlorophyll index (ChlRI) was $R^2 = 0.87$ ($p = 0.020$) for Krasnoufimskaya 100 variety and $R^2 = 0.81$ ($p = 0.018$) of Trizo increased. For Leningradskaya 97 variety, no relationship was found between the grain weight per plant and the chlorophyll content ($R^2 = 0.008$, $p = 0.88$).

For Krasnoufimskaya 100 and Trizo varieties at ONN, the leaf biomass increased with the ameliorant dose growth. The ameliorant application at the nitrogen deficiency led to an increase in the leaf biomass for the Krasnoufimskaya 100 variety, with the maximum value at 0.50 H_h (see Table 1). No significant effect of the ameliorant doses upon leaf biomass was identified in Trizo plants under nitrogen deficiency. It is characteristic that the doses of the ameliorant (0.25 and 0.75-1.00 H_h, respectively), causing in Krasnoufimskaya 100 and Trizo plants under optimal nitrogen nutrition the highest increase in the assimilating leaf area, leaf biomass, the chlorophyll content (ChlRI), and the grain yields, coincide.

Leaf area growth under the ameliorant application at optimal nitrogen nutrition resulted in a decrease in stems biomass, mostly for Krasnoufimskaya 100 variety (see Table 1). At nitrogen deficit, the stem biomass decreased insignificantly in response to the ameliorant application. The biomass of green ears of Krasnoufimskaya 100 and Trizo plants by the time of sampling was lower after the ameliorant application. These results show that the ameliorant, by optimizing plant nitrogen nutrition, promotes leaf growth and delays aging, which inhibits transition to heading and modulates ear growth. Similar processes can be observed with a high level of nitrogen nutrition. The formation of a powerful photosynthetic apparatus, able to keep up the photosynthesis activity for more prolonged time, gives undoubted advantages for realizing the potential of plants productivity. However, it may be supposed that the negative consequences of such changes, apparently, will be some elongation of the growing season and a tendency to lodging plants because of weaker stems.

2. Optical properties of wheat (*Triticum aestivum* L.) leaves as influenced by the ameliorant and nitrogen nutrition ((greenhouse trials, 4-fold replications)

Variant	Ameliorant dose, H_h	Nitrogen level	ChlRI	SIPI	R_{800}	PRI_{mod}	ARI_{mod}	FRI_{mod}
Krasnoufimskaya 100 variety								
1	0	ONN	0.530	1.019	30.90	0.462	0.557	3.797
2	0.50	ONN	0.557*	1.020	30.86	0.470	0.479*	4.586*
3	1.00	ONN	0.521	1.014*	30.25	0.472	0.496	3.152
4	0	DNN	0.525	1.014	30.39	0.445	0.455	3.737
5	0.50	DNN	0.537	1.014	31.91*	0.476*	0.502	2.982
6	1.00	DNN	0.543	1.012	32.25*	0.494*	0.487	3.636
Trizo variety								
7	0	ONN	0.590	1.009	31.84	0.445	0.428	3.561
8	0.50	ONN	0.590	1.008	32.26	0.448	0.443	2.855*
9	1.00	ONN	0.604*	1.011	33.67*	0.448	0.532*	2.551*
10	0	DNN	0.582	1.009	31.85	0.435	0.385	3.992
11	0.50	DNN	0.596	1.012	31.74	0.423	0.425	3.937
12	1.00	DNN	0.606*	1.011	32.89*	0.448	0.474	3.658

Note. ONN and DNN — optimal and deficit nitrogen nutrition. Reflection indexes: ChlRI — chlorophyll content, SIPI — carotinoids/chlorophyll ratio, R_{800} — light scattering within the leaf, PRI_{mod} — photochemical activity, characterizing intensity of heat dissipation, ARI_{mod} and FRI_{mod} — contents of anthocyanins and flavonols. For formulas to calculate the indexes, see the "Techniques" section. For statistical processing a nonparametric Wilcoxon test was applied with pairwise comparison of the variants 1 and 2, 1 and 3; 4 and 5, 4 and 6; 7 and 8, 7 and 9; 10 and 11, 10 and 12.

* Differences for corresponding pairwise comparison are statistically significant at $p \le 0.05$.

Optical characteristics of leaves of Krasnoufimskaya 100 and Trizo plants differ and vary depending on the nitrogen nutrition and the ameliorant doses (Table 2) with different sensitivity of the indexes to these factors. The maximum chlorophyll (ChlRI) content for both varieties was noted at the same ameliorant doses which were also necessary for the greatest increase in the grain yield (0.25

H_h for Krasnoufimskaya 100 and 0.75-1.00 H_h for Trizo).

Estimation of photosynthetic activity by the amount of chlorophyll does not always allow true assessment of the physiological state of plants. For example, in studying response of wheat and barley plants of different varieties to the action of ultraviolet radiation (λ = 280-380 nm), it was shown that a relationship between the reflection index of chlorophyll and the net production value is notable only under apparent growth inhibition [16-18]. Most likely, a small chlorophyll loss is aimed at creating conditions to restrict the effects of oxidative stress, and this is not always accompanied by growth inhibition and yield losses [16-18]. The obtained results allow us to conclude that in the early stages of mineral deficiencies, the intensity of photosynthesis and production process is not limited by a slight decrease in capacity of the photosynthetic apparatus. Apparently, one of the main reasons for the growth retardation is less effective conversion of light energy into chemical energy during photosynthesis. The effective use of light and fertilizers is currently of great interest for breeding, and non-invasive optical tools that allow breeders to quickly and quantitatively evaluate these parameters are among the most promising technologies [15, 24, 25].

Photochemical reflection index PRI was developed to estimate the rate of change in the relative content of xanthophyll cycle pigments which are active regulators of light flux in pigment-protein complexes [20, 26, 27]. Transformation of the carotenoids of xanthophyll cycle, proceeding with the heat generation, reduces the excess of chlorophyll absorption by the antenna complex at high light intensity or under stress conditions. Thermal dissipation of superfluous energy is the most important function of carotenoids in photoprotection of the chloroplast photochemical system from irreversible damage by large energy inflow into the reaction centers that cannot be used. The change in PRI during plant growth can be the result of combination of the xanthophyll cycle activity and the change in the total pool of chlorophylls and carotenoids, which is a response to long-term plant acclimatization to habitat [28, 29]. The tendency to an increase in thermal dissipation (PRI_{mod}) in response to the ameliorant application is most manifested in Krasnoufimskaya 100 variety, especially with a lower nitrogen level (see Table 2). We did not find any significant deviation in the photochemical reflection index in Trizo plants. At a lower nitrogen supply, the ameliorant facilitates the conversion of carotenoids and thermal dissipation, which is particularly manifested in Krasnoufimskaya 100 variety.

The content of carotenoids was also assessed by SIPI (see Table 2). The SIPI value remained practically unchanged, reliable ($p \leq 0.05$) differences were found only between variants 1 and 3. G.A. Blackburn [26] showed a nonlinear relationship between the SIPI value and the carotenoids to chlorophyll ratio, which is best described by the logarithmic model ($R^2 = 0.86$). SIPI is not sensitive enough at low ratios, but becomes more sensitive when they increase. Apparently, it can explain the lack of reliable SIPI changes in response to the ameliorant and reduction in the nitrogen availability, since the chlorophyll content in all the test variants was quite high.

A reliable ($p \leq 0.05$) decrease in ARI_{mod} and FRI_{mod} indices occurs only at high nitrogen nutrition, both in Krasnoufimskaya 100 and Trizo varieties. Anthocyanins and flavonols basically absorb radiation in green and ultraviolet spectra, slightly absorb in the red and almost do not absorb in the blue. Anthocyanins accumulation at stress reduces the flux of photosynthetically active radiation that penetrates chloroplasts, thus promoting protection of reaction centers in plastids at stress emergence [22, 30, 31]. There are data that anthocyanins and flavonols also perform antioxidant functions, in particular chloroplast-located flavonoids remove singlet oxygen forming in plant tissues under the action of various stressful envi-

ronmental factors [32].

The value of R_{800} primarily depends on the volume of intercellular air space, the mesophyll surface area to the leaf area ratio, as well as the internal structure of the leaf, the length of air-water boundary, size of cells and organelles [20]. Thus, an increase in R_{800} at mineral nutrition deficiency indicates a change in the inner structure of leaves, which promotes diffusion and a decrease in the absorbed solar radiation. Since the R_{800} value increases while the ameliorant introduction, it can be concluded that a decrease in soil acidity is accompanied by a change of leaf structure. Such changes in Krasnoufimskaya 100 variety occur when the ameliorant is introduced at nitrogen deficiency, whereas in Trizo variety at both high and low nitrogen levels in response to the ameliorant application at 1.0 H_h (see Table 2).

It was previously shown that one of the mechanisms of the nonspecific plant response to the stressful impact by downregulation of the photosystem II may be studied via changes of the reflection indices [16, 17]. Unlike the chlorophyll index the value of which characterizes the potential ability to absorb photosynthetically active radiation, all the other indices of Table 2 allow estimation of the absorbed light utilization efficiency. A slight decrease of this efficiency (with SIPI, R_{800}, PRI_{mod}, ARI_{mod} and FRI_{mod} increase) reflects inhibition of synthetic processes in the course of plants adaptation to the changing environmental conditions.

So, under good nitrogen supply (1 g/5 kg of soil), the grain weight in Leningradskaya 97 variety does not depend on the ameliorant application. In Krasnoufimskaya 100 there is a shift of positive response to the ameliorant towards lower doses, whereas Triso plants respond positively to all tested doses. The grain weight increase in these varieties at 0.25 H_h of the ameliorant is more than 30 and 17 %, respectively (for 0.50 and 0.75 H_h, the effect is also manifested, but 1.0 H_h reduces the grain yield of Krasnoufimskaya 100 plants). In the same varieties under different doses of the ameliorant the grain weight and the chlorophyll content in the leaves closely correlate ($R^2 = 0.87$ and $R^2 = 0.88$). The stimulating effect of the ameliorant is more pronounced at a lower dose of nitrogen (0.15 g/5 kg of soil). An increase in indices characterizing the efficiency of light energy conversion in photochemical processes (SIPI, R_{800}, PRI_{mod}, ARI_{mod} and FRI_{mod}) allows us to suggest that the ameliorant, by improving plant nutrition, promotes metabolism changes towards adaptation to environment conditions.

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THE MASS SPECTRAL ANALYSIS OF SOME CHEMICAL ELEMENTS' CONTENT IN THE FLAG LEAVES OF WHEAT (*Triticum aestivum* L.) ISOGENIC LINES WITH DIFFERENT RESISTANCE TO BROWN RUST

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Abstract

The wheat resistance to diseases, including the leaf rust pathogen, is the essential factor contributing to yield preservation. The Thatcher lines with *Lr*-genes are widely used in the assessment of differential interaction between resistance and virulence genes of a host plant and the pathogen. The aim of this work was a quantitative analysis of chemical element composition of flag leaves in 29 Thatcher isogenic lines with various genes for resistance to wheat leaf rust, Tc*Lr28*, Tc*Lr29*, Tc*Lr24*, Tc*Lr47*, Tc*Lr18*, Tc*Lr19*, Tc*Lr36*, Tc*Lr3ka*, Tc*Lr3bg*, Tc*Lr16*, Tc*Lr17*, Tc*Lr44*, Tc*Lr1*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr3a*, Tc*Lr10*, Tc*Lr11*, Tc*Lr14a*, Tc*Lr20*, Tc*Lr33*, Tc*Lr26*, Tc*LrB* (juvenile resistance); Tc*Lr35*, Tc*Lr12*, Tc*Lr21*, Tc*Lr48* (age-related resistance); Tc*Lr46*, Tc*Lr34* (partial resistance genes). The content of twenty-one chemical elements, including heavy, light metals, and metalloids (Na, Mg, Al, K, Ca, Cr, Mn, Fe, Co, Cu, Ni, Zn, Se, Mo, Ba, Pb, Sb, As, Cd, Be, Ag) in leaves of the isogenic lines was identified by mass spectrometry analysis. For quantitative analysis, leaves without visual symptoms of wheat leaf rust were collected. The disease development according to the R.F. Peterson's scale, pustule number on flag leaves, pustule area according to the ellipse area formula, and reaction type according to the scale of E.B. Mains and H.C. Jackson have been considered as parameters of the pathogenesis caused by the wheat leaf rust agent. The wide range of the parameters enabled us to use various statistical methods and to improve the accuracy of the differences identified. On the highly resistant lines protected by the *Lr*-genes of juvenile resistance, *Lr24, Lr28, Lr29, Lr47*, the signs of brown rust have not been revealed. The lines Tc*Lr18*, Tc*Lr19* and Tc*Lr36* were moderately resistant to the disease whereas the lines Tc*Lr3ka*, Tc*Lr3bg*, Tc*Lr12*, Tc*Lr16*, Tc*Lr17*, Tc*Lr44*, and Tc*Lr46* were moderately susceptible. The high susceptibility to the wheat brown rust was found for Tc*Lr1*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr3a*, Tc*Lr10*, Tc*Lr11*, Tc*Lr14a*, Tc*Lr20*, Tc*Lr33*, Tc*Lr26*, and Tc*LrB*. In the leaves of highly resistant isogenic lines with juvenile resistance and no symptoms, there was significantly less amount of heavy metals (Ni, Ag, Cr, Fe, Co, and Cd) and also K as compared to that in the lines with high susceptibility to the disease. The brown rust intensity decreased with the increase of the selenium content in the flag leaves. The lines bearing juvenile resistance *Lr*-genes showed a fewer reliable correlations between the chemical elements accumulation in the flag leaves and the greater affection by the leaf rust pathogen compared to the lines with genes of age-related resistance. It was found that the flag leaf levels of Al, Cr, Co, Sb, K in the lines with *Lr*-genes of juvenile resistance and Al, Fe, Ni, Zn in the lines with genes *Lr1, Lr10, Lr21, Lr3a, Lr24* significantly correlate with leaf rust manifestations. The leaf rust severity intensified significantly as the coefficients of Al, K, CR, Fe, Co, Ni, Sb, and Cd biological accumulation in the flag leaves increased. The line with *Lr34* gene, encoding a wheat protein similar to ABC transporters, differs from the other lines in lower biological accumulation coefficients for some toxic elements when compared to the lines which express plant NBS-LRR proteins. These findings can be helpful in spring soft wheat screening when breeding cultivars adapted to the environment conditions of the north-western regions of Russia.

Keywords: common spring wheat, isogenic Thatcher lines, *Lr* genes, elemental composition, pathogenesis, wheat brown rust

For successful crop production, conditions for growing plants, including spring soft wheat, must be optimal [1, 2]. Wheat yields are limited by abiotic and biotic stresses associated with both the phytosanitary state of agrocenoses and chemical pollution of the environment [3-6]. In farms with an unfavorable phytosanitary condition of spring wheat or in the presence of unfavorable lands in their surroundings, it is almost impossible to fully realize the achievements of plant breeding, seed production and advanced technologies [1]. Human activity affects composition and structure of species included in the natural and artificial biocenoses. Giant agrocenoses result in a sharp decrease in biodiversity, stimulate rapid evolution of pathogenic microorganisms, and lead to regular outbreaks of diseases [7, 8]. Chemical pollution of the environment affects yield and mechanisms of crop adaptation to environmental factors. Harmful chemical compounds may decrease yields of grain crops by 25-35 %, of fruit crops by 35-40 %, and of fodder plants by 35-50 % [9].

In recent years, considerable attention has been paid to absorption and accumulation of chemical elements and compounds by agricultural crops (10, 11). Although many elements (Zn, Cu, Mn, Mo, Co, Cr, Sn, V, Ni, etc.) in microdoses are essential, in high concentrations they become toxic. Some elements (Sb, As, Cd, Pb, Hg, Ag) are highly toxic in small quantities [12-14]. Wheat plants are particularly sensitive to the content of Mg, Cu, Mn, Zn, Mo, the lack of which disrupts the carbohydrate and nitrogen metabolism, as well as protein synthesis [15].

Brown rust caused by *Puccinia triticina* Erikss. is among the most dangerous diseases of wheat. Under favorable weather conditions and in a short period of time, the diseases can affect crops in vast areas, causing great damage to the grain yield [16, 17]. During evolution, plants developed protective mechanisms that ensure resistance to biotic and abiotic stresses [18]. Wheat resistance to diseases, including the causative agent of leaf rust, is the most important factor contributing to crop preservation [19]. At present, 77 *Lr* genes have been identified, of which 67 genes are mapped on chromosomes [7], and products of a number of *Lr* genes are also known. It has been found that the *Lr1*, *Lr10*, *Lr21*, *Lr3a*, and *Lr24* genes encode NBS-LRR proteins responsible for the recognition of *Avr* genes of phytopathogens [20-22], *Lr34* encodes a protein similar to the ABC transporters involved in the removal of toxic compounds from cells [23]. Despite the fact that plant resistance to pathogens is a genetically controlled trait, its manifestation is subjected to environmental influences and may be due to the heterogeneous structure of phytopathogen populations [24], deficiency or excess of macro- and microelements, and also depends on intake of phytotoxic elements into plants [2, 25-27].

It is impossible to assert with complete certainty that a particular nutrition can largely adapt a plant to various environmental conditions, weaken the disease or reduce its spread [27]. Thus, the diverse genotypes of wheat differ in their ability to efficiently absorb nutrients [28, 29]. The degree of development of pathogens from different groups (facultative and obligate parasites) depends on a certain ratio of chemical elements in plants [6]. With the development of pathogenesis in plants, basic physiological functions are disturbed, especially the movement of nutrients upward from the roots, the redistribution of chemical elements in organs, the utilization of elements. Moreover, in some organs there may be a lack of chemical elements, and in others — an overabundance up to toxic concentrations. In particular, cotton plants with symptoms of *Fusarium*

oxysporum f. *vasifectum* infection show an increase in amount of P in the leaves and a decrease in N, K, Ca, and Mg amounts [30].

This paper is the first report worldwide which identifies differences in the elemental composition of the Thatcher isogenic lines, varying in resistance to the brown rust pathogen. Our findings revealed the relationship between the leaf levels of a number of chemical elements and indicators of pathogenesis (intensity of the disease, the number of pustules, the area of pustules, and the type of response).

Our goal was a quantitative analysis of the elemental composition of the flag leaves of the Thatcher isogenic lines with different resistance to the wheat brown rust.

Techniques. In the test we used 29 isogenic Lr lines of spring soft wheat (*Triticum aestivum* L.) of Thatcher varieties with identified *Lr* genes: Tc*Lr28*, Tc*Lr29*, Tc*Lr24*, Tc*Lr47*, Tc*Lr18*, Tc*Lr19*, Tc*Lr36*, Tc*Lr3ka*, Tc*Lr3bg*, Tc*Lr16*, Tc*Lr17*, Tc*Lr44*, Tc*Lr1*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr3a*, Tc*Lr10*, Tc*Lr11*, Tc*Lr14a*, Tc*Lr20*, Tc*Lr33*, Tc*Lr26*, Tc*LrB* (juvenile); Tc*Lr35*, Tc*Lr12*, Tc*Lr21*, Tc*Lr48* (age-related); Tc*Lr46*, Tc*Lr34* (partial resistance genes) by courtesy of Research Institute of Plant Protection (St. Petersburg—Pushkin) and Federal Research Center the Vavilov All-Russian Institute of Plant Genetic Resources, (St. Petersburg). These lines are widely used in phytopathology to evaluate differential interaction of nonspecific plant resistance and the pathogen virulence [31]. Wheat varieties Leningradka (k-47882), Leningradskaya 97 (k-62935), Leningradskaya 6 (k-64900) were resistance standards.

The isogenic wheat lines of the Thatcher series were sown in 2014 (experimental field of VIR Pushkin Laboratory, Leningrad region). The area of the experimental plot for each sample was 1 m^2, the total number of plants on five rows of the experimental plot was 300 pcs. The elemental composition of flag leaves was assayed at the beginning of flowering, when the infectious process was in progress and it was still possible to find leaves without symptoms of pathogenesis on both resistant and susceptible varieties.

Na, Mg, Al, K, Ca, Cr, Mn, Fe, Co, Cu, Ni, Zn, Se, Mo, Ba, Pb, Sb, As, Cd, Be, and Ag levels in wheat leaves were evaluated by inductively coupled plasma mass spectrometry (ICP-MS, an ICP-MS 7700x mass spectrometer, Agilent Tech-Technologies, USA). The concentration of each element was determined by the average value of five repeated measurements with ICP-MS MassHunter software (Agilent Technologies, USA). Samples were mineralized in a Start D microwave digestion system in Teflon autoclaves (CEM, United States). Nitric acid was purified using DuoPUR system with sub-boiling distillation (Milestone, Italy). Deionized water with conductivity not less than 18.2 M was a solvent in all experiments. For measurement, samples (0.10 g) were exactly weighted using an analytical balance and placed in a Teflon autoclave with 5 ml of concentrated nitric acid added. The samples were subjected to decomposition in a microwave oven with temperature rise to 200 °C for 15 minutes, incubation at 200 °C for 15 minutes, and cooling to 45 °C. The dissolved sample was transferred to a 15 ml tube and diluted to 10 ml with deionized water. Aliquots of 1 ml were adjusted to 10 ml with 0.5 % nitric acid and used for analysis.

Soil elemental composition was assessed in samples from 10 randomized plots. Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, and Zn concentrations were estimated by mass spectrometry method as described hereinabove. Coefficients of biological accumulation, characterizing the degree of selective absorption of the element by plants, were calculated from the ratio of the element in plants to the soil.

Plant resistance to leaf rust was assessed by generally accepted phyto-

pathological indicators (the lesion intensity according to the Peterson scale and the type of reaction of Mains and Jackson) [32] and by additional parameters (the number of pustules and the area of a pustule calculated by the formula for the ellipse area) [33].

A complex of indicators of pathogenesis was determined for each line using 20 flag leaves collected at the beginning of flowering, at full flowering, and at the beginning of the grain milky ripeness. Phytopathological examination was performed by microscopy technique (MBS-10 stereo and monocular Mikromed R-1 microscopes, OOO Optical Devices, Russia).

The relationship between the content of chemical elements in wheat leaves and indicators of pathogenesis was estimated by parametric methods based on 95 % confidence intervals for mean values (*M*), standard errors of means (±SEM) with Student's t-test, and by non-parametric statistics method (Mann-Whitney test) as well as cluster analysis (*k*-means method) [34]. Statistical analysis was performed with the software packages SPSS 21.0, Statistica 6.0, and Excel 2013 [35, 36].

Results. Brown rust epiphytoty was recorded in 2014 in the spring soft wheat collection. The estimates of the damage to samples which were used as resistant standards were as follows: R_d (development of the disease) = 80±16 %, N_p (number of pustules) = 1594±824, S_p (area of pustules) = 0.096±0.014 mm^2, T (type of reaction) = 4 for Leningradka variety, R_d = 6±2 %, N_p = 54±19, S_p = 0.103±0.030 mm^2, T = 3 for Leningradskaya 97 variety, and R_d = 14±6 %, N_p = 255±183, S_p = 0.171±0.062 mm^2, T = 4 for Leningradskaya 6 variety. The Thatcher isogenic Lr lines with the *Lr24*, *Lr28*, *Lr29*, *Lr47* genes were high resistant (with reaction type 0) to the local population of wheat brown rust causative agent (Table 1).

1. Damage to wheat (*Triticum aestivum* L.) Thatcher isogenic lines with *Lr* genes caused by *Puccinia triticina* Erikss. under natural infection (St. Petersburg—Pushkin, 2014)

Line Thatcher with *Lr* gnes	Intensity of diseases, %	Number of pustules per leaf	Area of apustule, mm^2	Type of reaction, points
Tc*Lr28*	0.0	0.0	0.000	0
Tc*Lr29*	0.0	0.0	0.000	0
Tc*Lr24*	0.0	0.0	0.000	0
Tc*Lr47*	0.0	0.0	0.000	0
Tc*Lr19*	1.0±0.0	1.3±0.1	0.079±0.015	1
Tc*Lr18*	5.0±0.0	56.7±2.1	0.020±0.005	3
Tc*Lr35*	6.0±1.2	52.0±12.1	0.034±0.008	3
Tc*Lr48*	3.0±0.3	47.0±12.0	0.033±0.005	3
Tc*Lr17*	10.0±2.8	245.8±68.3	0.095±0.014	4
Tc*Lr44*	5.0±1.0	65.0±12.1	0.034±0.004	3
Tc*Lr46*	12.5±2.5	289.7±73.4	0.106±0.014	4
Tc*Lr3bg*	17.5±7.5	284.2±89.0	0.021±0.002	4
Tc*Lr3ka*	20.0±5.0	522.9±191.9	0.035±0.005	3
Tc*Lr16*	21.7±8.2	602.6±121.4	0.030±0.008	4
Tc*Lr12*	30.0±4.5	930.7±230.2	0.035±0.009	3
Tc*Lr21*	50.0±23.2	1198.7±334.2	0.038±0.008	3
Tc*Lr33*	50.0±12.1	629.2±41.2	0.056±0.016	4
Tc*Lr34*	52.5±12.5	922.5±231.2	0.085±0.014	4
Tc*Lr20*	64.0±12.3	1464.4±434.2	0.145±0.023	4
Tc*Lr2c*	70.0±5.0	928.8±119.6	0.035±0.009	4
Tc*Lr26*	75.0±25.0	1301.2±139.4	0.114±0.039	4
Tc*Lr1*	87.5±12.5	1345.4±91.0	0.028±0.007	4
Tc*Lr3a*	87.5±12.5	2388.4±271.6	0.069±0.011	4
Tc*Lr2b*	91.7±8.3	1043.7±329.8	0.079±0.029	4
Tc*Lr11*	100.0±0.0	2684.6±171.4	0.040±0.006	4
Tc*Lr10*	100.0±0.0	1259.4±194.9	0.056±0.006	4
Tc*Lr14a*	100.0±0.0	3247.3±91.7	0.061±0.007	4
Tc*LrB*	100.0±0.0	1337.0±182.7	0.053±0.014	4

On plants of the Thatcher line carrying the *Lr19* gene, isolated pustules

were observed, and the disease progression of did not exceed 1 %. In plants with genes *Lr18*, *Lr35*, Lr36, *Lr48*, the disease progression was from 1 to 3 % with the number of pustules from 1 to 57. The lines with genes *Lr17*, *Lr44*, *Lr46* (disease progression from 5 *to 13 %) were relatively resistant. In plants with genes Lr3ka, Lr3bg, Lr12, Lr16, the progression of the disease was 18-30 %. The lines with Lr1, Lr2c, Lr2b, Lr3a*, *Lr10*, *Lr11*, *Lr14a*, *Lr20*, *Lr21*, *Lr26*, *Lr33*, *Lr34*, *LrB* showed high lesion (of 50 to 100 %).

It should be noted that in recent years, the frequency of brown rust isolates virulent to the Tc*Lr3a*, Tc*Lr3bg*, Tc*Lr3ka*, Tc*Lr11*, Tc*Lr12b*, Tc*Lr16*, Tc*Lr17*, Tc*Lr18* lines in the North-West Russia was high and reached 80-100 %. The Tc*Lr1*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr15* and Tc*Lr26* lines showed high virulence polymorphism from 38 to 100 %. That is, the trend towards an increase in the frequency of isolates virulent to Tc*Lr1* observed since the beginning of the 2000s, continues [24].

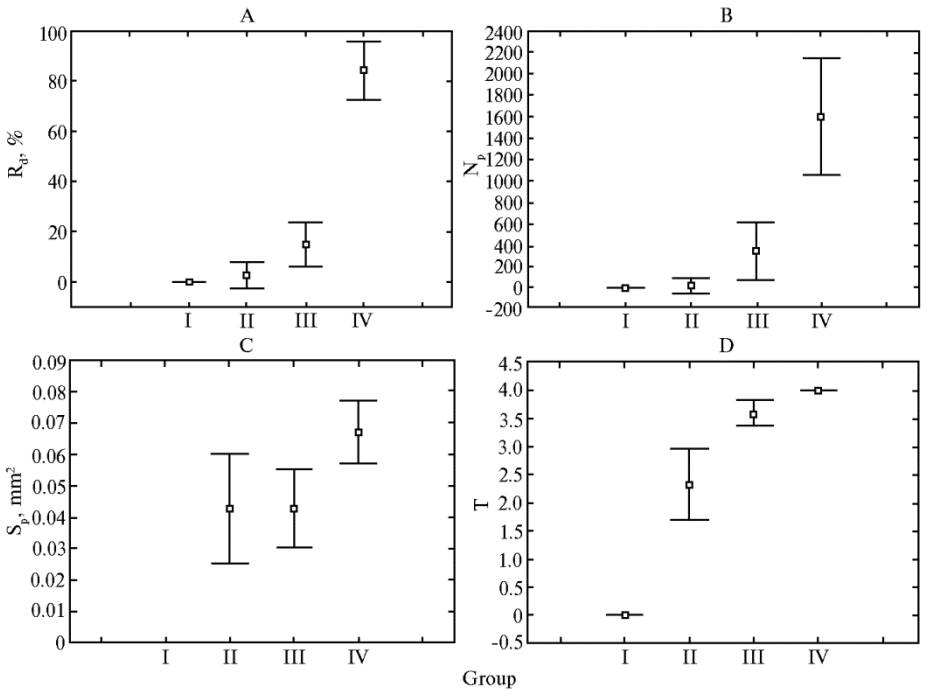

Fig. 1. Damage caused by *Puccinia triticina* Erikss. to wheat (*Triticum aestivum* L.) Thatcher isogenic lines with juvenile *Lr* genes: A — disease development (group I of high resistance, the lines with no symptoms; group II of moderate resistance at $R_d = 2.5 \pm 1.2$ %; group III of moderate sensitivity at $R_d = 14.8 \pm 3.2$ %; group IV of high sensitivity at $R_d = 84.2 \pm 5.2$ %); B — number of pustules (group I with no signs of the diseases; group II at $N_p = 22.0 \pm 7.5$; group III at $N_p = 344.1 \pm 97.5$; group IV at $N_p = 1602.7 \pm 244.4$); C — pustule area (group I with no signs of the diseases; group II at $S_p = 0.043 \pm 0.018$ mm^2; group III at $S_p = 0.043 \pm 0.013$ mm^2; group IV at $S_p = 0.067 \pm 0.011$ mm^2); D — type of the reaction (group I with no signs of the diseases; group II at $T = 2.3 \pm 0.7$; group III at $T = 3.6 \pm 0.2$; group IV at $T = 4.0 \pm 0.0$). The graphs show average values and 95 % confidence intervals (St. Petersburg—Pushkin, 2014).

Clustering (*k*-means method) divides the carriers of *Lr* genes of juvenile resistance to brown rust pathogen into four groups (Fig. 1). The highly resistant Thatcher lines (Tc*Lr28*, Tc*Lr29*, Tc*Lr24*, Tc*Lr47*) without symptoms of brown rust ($R_d = 0$ %) during the vegetative period are assigned to group I. The moderately resistant Thatcher lines (Tc*Lr18*, Tc*Lr19*, Tc*Lr36*) forme group II. In group II, the damage from the causative agent of brown rust is significantly lower during all phases of wheat growth compared to moderately susceptible lines Tc*Lr3ka*, Tc*Lr3bg*, Tc*Lr16*, Tc*Lr17* of group III and highly susceptible lines

Tc*Lr1*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr3a*, Tc*Lr10*, Tc*Lr11*, Tc*Lr14a*, Tc*Lr20*, Tc*Lr33*, Tc*Lr26*, Tc*LrB* of group IV.

As the resistance of isogenic lines to brown rust is commonly evaluated by lesions caused by the pathogen on wheat flag leaves, we determined the elemental composition of flag leaves. The use of a set of indicators increased the number of statistical data analysis methods and improved the accuracy of identifying differences between the leaves in elemental composition.

Comparison of the average amounts of the elements by Student's *t*-test showed that the Thatcher group of highly resistant lines with no signs of brown rust (Tc*Lr24*, Tc*Lr28*, Tc*Lr29*, Tc*Lr47*) differ from the combined group of moderately resistant, moderately susceptible and highly susceptible lines in a number of indicators. The high resistant lines have significantly lower levels of K (by 21.41 %), Ni (by 56.54 %) (at $P < 0.05$). A nonsignificant decrease occurs in the amounts of Ca (by 10.64 %), Mg (by 10.06 %), Al (by 14.18 %), Cr (by 44.24 %), Mn (by 1/41 %), Fe (by 9.03 %), Co (by 11.34 %), Pb (by 0.25 %), Sb (by 23.40 %), Cd (by 18.23 %), Be (by 28.03 %), and Ag (by 35.97 %). The differences are not significant ($P > 0,05$) for microelements Na (by 33.04 %), Cu (by 6.73 %), Zn (by 4.65 %), Se (by 15.16 %), Mo (by 3.07 %), Ba (by 3.31 %), and As (by 5.56 %) most of which are essential for plant growth and development.

Calculation of 95 % confidence intervals for means *M* (Fig. 2) revealed that highly resistant Thatcher lines (group I) contain significantly lower K, Cr, Fe, Co, Ni, Cd, and Ag levels compared to highly susceptible (group IV). Differences appear between moderately resistant group II and moderately susceptible group III in Ni concentration, and between groups III and IV in Cr, Fe, Co, Ni, and Ag concentrations.

Parametric statistics methods showed that plants of group I have significantly lower Co content (Mann-Whitney test $U = 7.0$ at $P = 0.04$) and Ni ($U = 5.0$ at $P = 0.03$) than iof group IV. Moderately resistant isogenic lines (group II) had significantly lower Ni level as compared to highly susceptible lines of group IV ($U = 3.0$ and $P = 0.04$). Lines with moderate susceptibility to brown rust (group III) show significantly lower levels of Sb ($U = 7.0$ at $P = 0.02$), Ca ($U = 10.0$ at $P = 0.02$), Co ($U = 9.0$ at $P = 0.03$), and Pb ($U = 10.0$ at $P = 0.03$) compared to those in group IV.

The non-parametric analysis of Spearman correlations (at $P < 0.05$) indicates that the brown rust damage to isogenic Thatcher lines with juvenile *Lr* genes increases significantly with a rise in concentrations of Al ($r = 0.6$ for R_d), Cr ($r = 0.5$ for R_d and $r = 0.5$ for N_p), Co ($r = 0.6$ for R_d and $r = 0.5$ for N_p), Ni ($r = 0.7$ for R_d, $r = 0.6$ for N_p and $r = 0.5$ for T), Sb ($r = 0.5$ and $r = 0.4$ for N_p), and K ($r = 0.6$ for T).

The lesion intensity in Thatcher isogenic lines with *Lr* genes for age-related resistance significantly decreases with an increase in leaf Se ($r = –0.91$ for R_d and $r = –0.89$ for N_p), in contrast to higher damage in lines with juvenile *Lr* genes ($r = –0.11$ for R_d, $r = –0.13$ for N_p and $r = 0.34$ for S_p).

In isogenic lines with juvenile *Lr* genes of resistance ($R_d = 43.80 \pm 8.67$ %; $N_p = 844.17 \pm 194.90$; $S_p = 0.046 \pm 0.007$ mm^2), there are significantly lower ($P < 0.05$) leaf levels of Be (by 21.41 %; Student's *t*-test $T_{05} = –2.17$; Mann-Whitney test $U = 11$) compared to the lines carrying *Lr* genes of age-related resistance ($R_d = 22.25 \pm 9.05$ %; $N_p = 557.10 \pm 200.12$; $S_p = 0.035 \pm 0.001$ mm^2). Insignificantly lower ($P > 0.05$) amounts are detected for Cd (by 22.12 %), Ag (by 21.66 %), Cr (by 16.86 %), Pb (by 13.67 %), Co (by 2.67 %), Mo (by 2.45 %), Na (by 1.85 %), and Al (by 1.7 %). And finally, unreliably higher ($P > 0.05$) concentrations are found for Fe (by 1.51 %), Sb (by 1.73 %), Ca (by 1.74 %), Mn (by 4.33 %) Ba (by 4.46 %), As (by 4.72 %), Se (by 5.91 %), Zn (by 7.64 %), Mg

(by 19.58 %), K (by 21.31 %), Ni (by 35.58 %), and Cu (by 54.03 %).

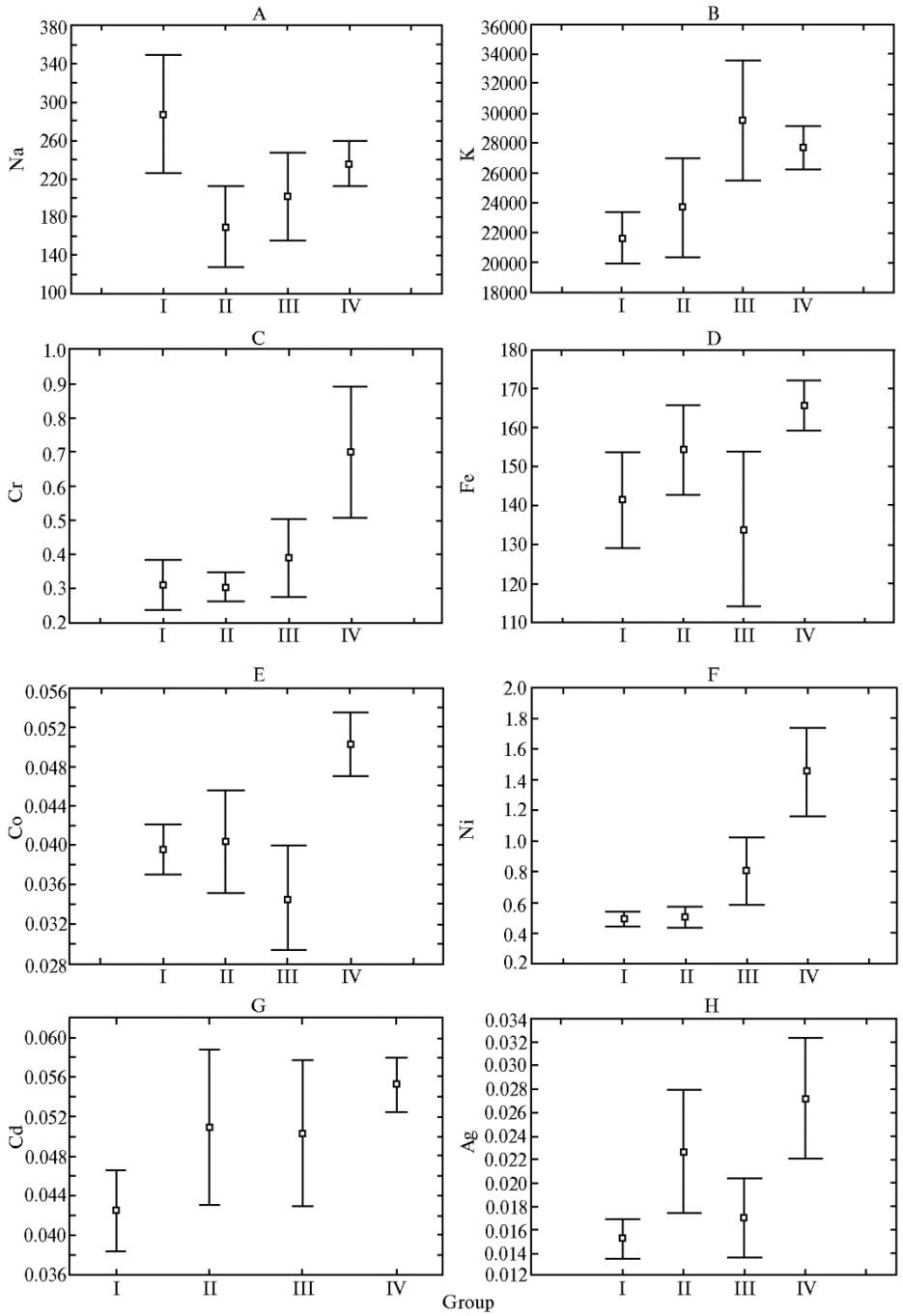

Fig. 2. Content of microelements in leaves of wheat (*Triticum aestivum* L.) isogenic lines Thatcher with juvenile *Lr* genes: A — Na, B — K, C — Cr, D — Fe, E — Co, F — Ni, G — Cd, H — Ag (group I of high resistance, the lines with no symptoms; group II of moderate resistance at R_d = 2.5±1.2 %; N_p = 22.0±7.5; S_p = 0.043±0.018 mm^2; T = 2.3±0.7; group III of moderate sensitivity at R_d = 14.8±3.2 %; N_p = 344.1±97.5; S_p = 0.043±0.013 mm^2; T = 3.6±0.2; group IV of of high sensitivity at R_d = 84.2±5.2 %; N_p = 1602.7±244.4; S_p = 0.067±0.011 mm^2; T = 4.0±0.0). The graphs show average values, μg/g, and 95 % confidence intervals (St. Petersburg—Pushkin, 2014).

Analysis of the matrix of mutual correlations of the wheat leaf elemental

composition shows that a group of Thatcher isogenic lines with juvenile *Lr* genes of resistance have less reliable Spearman and Pearson correlation coefficients (by 65.34 and 74.22 %, respectively) compared to the lines with genes of age-related resistance.

In the isogenic lines with juvenile *Lr* genes, when compared to the lines with *Lr* genes of partial field resistance (R_d = 32.50±10.05 %; N_p = 606.06±316.40; S_p = 0.095±0.010 mm^2), the leaf concentrations are significantly higher ($P < 0.05$) for Ca (by 53.47 %), Al (by 55.38 %), Mg (by 85.76 %), though increased values are unreliable ($P > 0.05$) for Pb (by 6.03%), Zn (by 6.78 %), Mn (by 7.68 %), K (by 9.67 %), Be (by 10.14 %), As (by 15.40 %), Co (by 16.46 %), Fe (by 17.89 %), Cd (by 29.04 %), Sb (by 51.60 %), Mo (by 56, 90 %), and Ni (by 107.56 %). A decrease in values is unreliable ($P > 0.05$) for Cu (by 49.84 %), Ag (by 25.58 %), Cr (by 19.82 %), Ba (by 13.19 %), Na (by 3.93 %), and Se (by 3.14 %).

In the isogenic lines with *Lr* genes of age-related resistance, when compared to the lines with *Lr* genes of partial field resistance, there is an unreliably lower ($P > 0.05$) content of Cu (by 67.44 %), Ba (by 16.19 %), K (by 11.07 %), Se (by 8.55 %), Ag (by 5.0 %), Cr (by 3.55 %), Na (by 2.12 %), Zn (by 0.80 %) and unreliable higher ($P > 0.05$) content of Mn (by 3.21 %), As (by 10.20 %), Fe (by 16.13 %), Co (by 19.66 %), Pb (by 22.83 %), Sb (by 49.01 %), Ca (by 50.85 %), Ni (by 53.09 %), Mg (by 55.3 %), Al (by 58.07 %), Mo (60.12 %), Cd (65.7 %), and Be (147.22 %).

Nonparametric correlation analysis determined the relationship between the leaf elemental composition of the Thatcher isogenic lines with *Lr1*, *Lr10*, *Lr21*, *Lr3a*, *Lr24* genes encoding plant NBS-LRR proteins (20-22) and the indicators of wheat rust development (Table 2). It was established that higher levels of Al, Fe, Ni, and Zn are associated with more intensive development of wheat brown rust (the values of the Spearman correlation coefficient were reliable).

2. Correlation between the lesion of wheat (*Triticum aestivum* L.) isogenic lines Thatcher with *Lr1*, *Lr10*, *Lr21*, *Lr3a*, *Lr24* genes by brown rust pathogen (*Puccinia triticina* Erikss.) and the leaf elemental composition (St. Petersburg—Pushkin, 2014)

Element	R_d	N_p	S_p	T
Na	0.31	–0.10	–0.50	0.22
Mg	0.87	0.50	0.30	0.78
Al	0.95*	0.56	0.41	0.86
K	0.46	0.20	–0.30	0.45
Ca	0.87	0.60	0.60	0.78
Cr	–0.72	–0.60	–0.10	–0.78
Mn	0.41	0.00	–0.30	0.34
Fe	0.97*	0.60	0.50	0.89*
Co	0.46	0.50	0.60	0.45
Cu	0.67	0.20	0.70	0.45
Ni	0.82	0.70	0.30	0.89*
Zn	0.56	0.90*	0.50	0.78
Se	0.62	0.30	0.50	0.45
Mo	–0.56	–0.80	–0.20	–0.78
Ba	–0.21	–0.70	–0.70	–0.45
Pb	–0.41	0.00	0.30	–0.34
Sb	0.55	0.21	–0.10	0.52
As	–0.41	0.10	–0.60	–0.11
Cd	0.21	0.10	0.10	0.22
Be	0.16	–0.16	0.00	0.06
Ag	–0.36	0.20	0.20	–0.11

N o t e. R_d — disease development, %; N_p — number of pustules; S_p — the area of a pustule, mm^2; T — type of the response, points.
* Spearman correlation coefficients are statistically significant ($P < 0.05$).

We used the ratio of leaf level to soil level for each element to calculate the bioaccumulation coefficients which characterize selective absorption of the element by plants. It was turned out that the pathogen becomes significantly

more aggressive as the coefficients of Al, K, Cr, Fe, Co, Ni, Sb and Cd accumulation in flag leaves grow (Table 3).

In contrast to Thatcher lines with genes *Lr1*, *Lr10*, *Lr21*, *Lr3a*, *Lr24*, in the line with *Lr34* gene encoding a wheat protein similar to ABC transporters involved in detoxication processes [23], the bioaccumulation coefficients are reliably lower ($P < 0,05$) for Cr (by 75.1 %) and Ni (by 49.5 %), unreliably lower ($P > 0.05$) for Na (by 15.6 %), Mg (by 38.0 %), Al (by 41.2 %, K (by 22.9 %), Ca (by 45.1 %), Mn (by 8.4 %), Fe (by 17.5 %), Co (by 98.5%), Zn (by 12.7 %), Mo (by 47.9 %), Pb (by 13.3 %), and Cd (by 37.5 %) vs. a significant increase in Cu (by 50.3 %, $P < 0.05$). Additionally, in the line Tc*Lr34* compared to Tc*Lr1*, Tc*Lr10* and Tc*Lr3a*, the infection is less intensive and the number of pustules on flag leaves is smaller (by 66.7 % and 45.8 %; by 90.5 % and 36.5 %; and by 66.8 % and 158.9 %, respectively; $P < 0.05$).

3. Correlation between bioaccumulation coefficients of chemical elements and brown rust infection (*Puccinia triticina* Erikss.) intensity in wheat (*Triticum aestivum* L.) isogenic lines Thatcher (St. Petersburg—Pushkin, 2014)

Element	R_d	N_p	S_p	T
Na	0.11	0.07	–0.18	0.12
Mg	0.20	0.13	–0.14	0.11
Al	0.47*	0.43*	–0.04	0.30
K	0.42	0.38*	0.24	0.49*
Ca	0.27	0.21	–0.02	0.13
Cr	0.44*	0.43*	0.25	0.32
Mn	–0.02	–0.02	–0.05	0.00
Fe	0.39*	0.38*	0.07	0.27
Co	0.49*	0.46*	0.10	0.35
Cu	0.30	0.33	0.27	0.23
Ni	0.65	0.64*	0.14	0.51*
Zn	–0.02	–0.02	0.01	0.02
Se	–0.09	–0.19	–0.19	–0.04
Mo	0.21	0.19	0.08	0.05
Ba	0.23	0.13	0.14	0.18
Pb	0.03	0.08	–0.20	–0.05
Sb	0.43*	0.40*	–0.07	0.23
As	0.05	0.15	0.09	0.06
Cd	0.38*	0.41*	0.14	0.24
Be	–0.05	–0.03	–0.27	–0.13
Ag	0.16	0.19	0.29	0.27

N o t e. R_d — disease development, %; N_p — number of pustules; S_p — the area of a pustule, mm^2; T — type of the response, points.
* Spearman correlation coefficients are statistically significant ($P < 0.05$).

Interestingly, the Thatcher lines with juvenile resistance genes *Lr28*, *Lr29*, *Lr24* and *Lr47* without signs of brown rust during vegetation period have reliably lower accumulation of heavy metals Ni, Ag, Cr, Fe, Co, Ni, Cd, and also K, which is in line with our findings reported for other wheat varieties and lines [37]. Perhaps, this is due to probable phytotoxicity of heavy metals at higher leaf concentrations, which leads to phytoimmunity weakening [38] and more losses, especially in susceptible varieties [39]. Excessive amount of potassium in the leaves delays the sodium input into the plant, causing metabolic disturbances and growth retardation. In laboratory tests, it was shown [40] that application of potassium chloride in seedling growing significantly increases (4.9-fold) the efficiency of inoculum production of brown rust pathogen.

In our tests, the intensity of brown rust pathogen development decreased with an increase in the selenium content in flag leaves of Thatcher isogenic lines. As known, Se has a positive effect on wheat growth and photosynthesis indicators [41]. This tendency is most characteristic of lines with Lr genes of age-related resistance.

The Thatcher isogenic lines with *Lr* genes of juvenile resistance have fewer reliable correlations between the flag leaf levels of chemical elements and

greater brown rust damage than the lines with genes for age-related resistance. This was confirmed by parametric and non-parametric Pearson and Spearman coefficients (at $P < 0.05$). The revealed peculiarities of correlations indicate a delicate tuning of biochemical processes in switching-on the host plant defense mechanisms against pathogens.

We found significant positive correlations between the damage to wheat plants caused by brown rust and the flag leaf levels of Al, Cr, Co, Sb, and K for the lines with juvenile resistance *Lr* genes, and of Al, Fe, Ni, Zn for the lines with *Lr1*, *Lr10*, *Lr21*, *Lr3a*, and *Lr24* genes. The intensity of the brown rust pathogen development significantly rises with an increase in the coefficients of Al, K, Cr, Fe, Co, Ni, Sb, and Cd biological accumulation in flag leaves.

Thus, as per our findings, the elemental profile of the Thatcher spring wheat soft wheat series, which is due to the genetically determined resistance and agrochemical conditions, can influence the intensity of brown rust pathogen development. The disease becomes more sever as the leaf amount of toxic elements increases, including heavy metals. These results can be used in breeding spring soft wheat varieties adapted to the environmental conditions of the North-West Russia.

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GENETIC STRUCTURE OF RUSSIAN AND KAZAKHSTANI LEAF RUST CAUSATIVE AGENT *Puccinia triticina* Erikss. POPULATIONS AS ASSESSED BY VIRULENCE PROFILES AND SSR MARKERS

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Abstract

Leaf rust caused by *Puccinia triticina* Erikss. is an economically significant disease of spring wheat in the West-Asian Russia and Northern Kazakhstan. Successful wheat breeding for leaf rust resistance necessitates characterization of *Lr* gene effectiveness, the impact of new wheat varieties on the pathogen virulence, and isolation between populations of *P. triticina*. Until now, nobody used a uniform infectious material in *P. triticina* population study, as it was collected from a different set of varieties in each region. Thence, the virulence assessment data were significantly influenced by the effects of host plants. We were the first to compare the structure of *P. triticina* populations in the West-Asian Russia and Northern Kazakhstan on virulent and molecular genetic properties, using as a source of infectious material a single set of the wheat samples dated 2016 that were tested there within the framework of the Kazakhstan-Siberian Spring Wheat Improvement Network Program (KASIB). Ninety one single-pustule isolates have been tested in the virulence analysis, including 13 isolates from Chelyabinsk, 28 from Omsk, 6 from Kurgan, 16 from Akmolinskaya, 16 from Pavlodarskaya, and 12 from Karagandinskaya provinces. Eleven phenotypes of virulence have been identified with the use of 20 isogenic Thatcher lines with *Lr* genes (Tc*Lr*). The similar phenotypes were found on cultivars Duat, Tertsia, Omskaya 35, Pamyati Azieva, Saratovskaya 29, Chebarkulskaya 3 and line Eritrosperum 85-08. The Russian pathotypes found on variety Astana and lines Lutescens 1003 and Lutescens 6/04-4 differed from Kazakhstan ones in virulence to Tc*Lr11*, and those colonizing variety GVK 2074/4 and line Lutescens 715 differed in avirulence to Tc*Lr18*. More significant differences in the virulence rang between regional populations have been observed on the line Lutescens 34/08-1 and the variety Rodnik. All studied *P. triticina* isolates were avirulent to Tc*Lr19*, Tc*Lr24* and virulent to Tc*Lr3a*, Tc*Lr3bg*, Tc*Lr3ka*, Tc*Lr14a*, Tc*Lr14b*, Tc*Lr16*, Tc*Lr17*, Tc*Lr30*. The variation in virulence frequencies was observed on the wheat lines with the genes *Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr9*, *Lr15*, *Lr18*, *Lr20*, and *Lr26*. Virulence to *Lr9* was found in the pathogen populations collected from the wheat samples Duet, Tertsia, Chebarkulskaya 3, Lutescens 34/08-19 carrying this gene. The differences in populations on virulence were found using the indices of genetic distances Nei D and Fst, the analog for binary data in AMOVA. The Nei index values indicated a

high similarity between the majority of the Northern-Kazakhstani and Russian populations, except of those from Chelyabinskaya and Karagandinskaya provinces. According to the Fst index, the high similarity was found between the populations from Omsk, Kurgan and Northern Kazakhstan. The Chelyabinsk population appeared to be close to the Kurgan but varied from others. In the test, a total of 46 isolates and 9 genotypes have been identified using 12 SSR markers. In this, 21 polymorphic alleles were found in the studied set. The Nei and Fst indices revealed the moderate differences between the Chelyabinsk and Karaganda populations and a high similarity between other populations studied. The current survey defined the high similarity of leaf rust pathogen populations in West-Asian Russia and Northern Kazakhstan, assuming the existence of a single fungus population in the studied territories. In order to prevent the *P. triticina* epiphytoties on the adjacent territories of Russia and Kazakhstan, the constant updating of wheat varieties and higher genetic diversity are strongly recommended. In addition, the varieties should be grown according to the «mosaic distribution» scheme using optimal areas for genetically homogeneous varieties.

Keywords: *Puccinia triticina* Erikss., leaf rust, common wheat, populations, virulence, *Lr*-genes, SSR markers

Spring wheat is the main cereal crop in the Western Siberia, the Urals and Northern Kazakhstan. Leave rust (causal agent *Puccinia triticina* Erikss.) is the most prevailing and harmful disease limiting wheat yields in these regions [1-3]. With its epiphytotic development, wheat losses can reach 15-30 % [4].

Most of productive varieties cultivated in Western Siberia, the Urals and Northern Kazakhstan are susceptible to leaf rust with varying degree [2, 3]. Shuttle breeding within the framework of the CIMMYT (International Maize and Wheat Improvement Center) Kazakhstan-Siberian Spring Wheat Improvement Network Program (KASIB), leverages global genetic resources to improve the carried out breeding programs efficiency, including on leaf rust resistance [1, 5].

Strategies for genetic wheat protection are based on the creation and introduction of leaf rust resistant varieties, which requires data about the resistance genes efficiency to its causal agent (*Lr*-genes), virulence changes in the pathogen populations under the influence of new varieties, isolation between populations and the potential genes flow between them. In structure of pathogen population, frequency of virulence genes is the most important criterion, but does not always characterize it adequately [7, 8], because the concentration of virulence genes in the fungus population is closely linked to the selective pressure of cultivated wheat varieties. Despite this, in Russia and abroad most population studies of the causatvel agent of leaf rust were performed on contagious material collected from a different set of varieties in each region [9, 10].

Application of the selectively neutral molecular markers to compare DNA polymorphisms of a pathogen makes it possible to refine and to add the results of virulence analysis. Such markers are indispensable in the pathways researches of organism migration, formation of the uniform epidemiologic regions and emergence of areas [7]. Molecular markers (RAPD — random amplification of polymorphic DNA, AFLP — amplified fragment length polymorphism, SSR — simple sequence repeat) have been involved in *Puccinia triticina* study since the mid-1990s [11], with the most extensive use of microsatellites. Molecular markers were used to describe the structure of *P. triticina* populations in the North and South American continents, in Western Europe, in the Middle East, Central Asia and the North Caucasus [4, 8, 12].

We were the first to use infectious material which was collected from a single set of wheat varieties at several geographical locations, which reduces the selective influence of host plants on the results of virulence evaluation. In this case, a high population similarity to the causative agent of leaf rust in the Urals, Western Siberia and Northern Kazakhstan is identified, which confirmes the suggestion of the uniform fungus population in these territories.

The aim of the work is to analyze the genetic structure of *Puccinia triticina* populations in the West-Asian Russia and Northern Kazakhstan for viru-

lence and microsatellite loci.

Techniques. Samples of *Puccinia triticina* Erikss. Were collected in 2016 on 15 wheat varieties, which were studied within the frame of the KASIB program in the Chelyabinsk, Omsk, Kurgan regions (Russia) and Akmolinskaya. Karagandinskaya, Pavlodarskaya regions (Kazakhstan). In 2016, in all these regions, the weather conditions was favorable for leaf rust development, prevalence of the infection among the varieties varied from 0 to 100 %.

The isolates were cultured by laboratory method as per the description [13]. Virulence of 91 single-pustule isolates (2-3 from each studied wheat sample) was analyzed with the use of 20 almost isogenic Thatcher lines with *Lr* genes (Tc*Lr*). For this, 3 grains of each Tc*Lr* line were seeded in the soil. Seedlings (10-14-day old, leaf 1 phase) were inoculated with spore suspension (10^6/ml) and placed in a Versatille Environmental Test Chamber MLR-352H (SANYO Electric Co., Ltd., Japan) at 22 °C and 75 % humidity. On day 10 the results were estimated according to E.B. Mains and H.S. Jackson scale range [14]: 0 — no signs, 0; — necrosis without pustules, 1 — very small pustules surrounded by necrosis, 2 — medium size pustules, surrounded by necrosis or chlorosis, 3 — medium size pustules without necrosis, 4 — large pustules without necrosis, X — different types of pustules on one leaf, chlorosis and necrosis are presented (plants with the X type reaction considered as susceptible).

Virulence phenotypes were classified according to the North American system [15]. For this purpose, 20 Tc*Lr* lines were divided into five sets: 1 — Tc*Lr1*, Tc*Lr2a*, Tc*Lr2c*, Tc*Lr3a*; 2 — Tc*Lr9*, Tc*Lr16*, Tc*Lr24*, Tc*Lr26*; 3 — Tc*Lr3ka*, Tc*Lr11*, Tc*Lr17*, Tc*Lr30*, 4 — Tc*Lr2b*, Tc*Lr3bg*, Tc*Lr14a*, Tc*Lr14b*; 5 — Tc*Lr15*, Tc*Lr18,* Tc*Lr19*, Tc*Lr20*. The alphabetic phenotype code was determined using the software package VAT (Virulence Analysis Tool; https://en-lifesci.tau.ac.il/profile/kosman/vat). Indices of interpopulation differences by M. Nai (Nei D) and Φ_{PT} (analog of F_{st} for binary data in AMOVA, analysis of molecular variance) were calculated using the GenAlEx (Genetic analysis in Excel 6.5; http://biology.anu.edu.au/GenAlEx).

A total of 46 single pustule *P. triticina* isolates, including Chelyabinskaya 7, Kurganskaya 5, Omskaya 14, Akmolinskaya 8, Pavlodarskaya 8 and Karagandinskaya 4 (1-2 isolates from each studied wheat sample) were used for SSR analysis. Procedure of pathogen collection was similar to described by J.A. Kolmer et al. [4]. DNA was isolated according to A.F. Justesen et al. technique [16]. The polymorphism of 12 microsatellite loci (PtSSR50, PtSSR55, PtSSR61, PtSSR91, PtSSR92, PtSSR151, PtSSR152, PtSSR158, PtSSR161, PtSSR164, PtSSR173, and RB35) was determined. The used SSR markers are designed to assess genetic diversity of *P. triticina* [4, 8, 10, 12]. PCR protocols and primer sequences are presented in the original papers [17, 18] (ABI Prism 3500 genetic analyzer, Applied Biosystems, USA; Hitachi, Japan). The SSR allele sizes were determined with GeneMapper 4.1 software. To assess the effect of cultivated varieties on the pathogen virulence, *Lr* genes were identified in wheat samples. Markers of genes *Lr1* (WR003), *Lr9* (SCS5), *Lr10* (FI.2245/*Lr10-6*/r2), *Lr26* (SCM 9) and *Lr34* (L34DIN9/Lr34Plus) were used [19]. PCR protocols and primers for marking varieties and lines were as per original works cited by A. Serfling et al. [19], the PCR was carried out with a C1000TM Thermal Cycler (Bio-Rad, USA).

Statistical results processing of SSR analysis, including P calculation, was carried out with GenAlEx 6.5 software (genetic analysis in Excel, 6.5; http://biology.anu.edu.au/GenAlEx). Intrapopulation genetic diversity of microsatellite loci in fungi was described by the following indices: the mean number of alleles per locus (Na), number of effective alleles (Ne), expected (H_e) and

1. Wheat varieties infected by *Puccinia triticina* Erikss., with identified phenotypes of virulence and SSR genotypes of the pathogen in different geographic populations (2016)

Sample	Russia						Kazakhstan					
	Chelyabinskaya Province		Omskaya Province		Kurganskaya Province		Akmolinskaya Province		Pavlodarskaya Province		Karagandinskaya Province	
	I, %	P/SSR	I, %	P/SSR	I, %	P/SSR	I, %	P/SSR	I, %	P/SSR	I, %	P/SSR
Astana2	50S	–	40S	TGTTR/D	70S	TGTTR/D	90S	TGPTR/D	50S	TGPTR/G	45S	–
ГВК 2074/4	100S	–	80S	THTTM/D	70S	–	50S	–	20S	THTTR/B	10S	THTTR/A
Duat	50S	PQTKH/F	50S	PQTKH/F	–	–	–	–	–	–	–	–
Lutescens 1003	20MS	–	100S	TGTTR/F	40S	–	90S	TGPTR	50S	TGPTR/G	30S	–
Lutescens 1003	10MR	–	5MR	–	20S	PQTKH/B	5S	TQTTR/B	20S	–	0	–
Lutescens 1003	50S	THTTR/B	10MR	THTTR/B	10S	–	5S	THPTR/B	20S	THPTR/B	0	–
Lutescens 1003	50S	–	100S	TGTTM/H	60S	–	70MS	TGTTR/B	50S	–	20S	TGTTR/C
Omskaya 35	50S	–	100S	TGTTR/F	70S	TGTTR/G	70S	TGTTR/C	50S	–	–	–
Memory of Aziev	50S	–	100S	TGTTR/C	80S	TGTTR/C	90S	TGTTR/C	40S	–	40S	–
Rodnik	20S	THTTR/A	100S	TGTTR/A	30S	–	50S	TGTTR/F	30S	MGTKH/B	10S	–
Saratovskaya 29	50S	–	100S	TGTTR/F	50S	–	100S	–	30S	TGTTR/F	70S	–
Stepnaya 53	80S	–	80S	TGTTM/B	60S	–	50S	–	50S	TGTTM/B	5MS	TGPTR/C
Terzia	20MS	–	40S	–	60S	TQPTR/B	90S	–	40S	TQPTR/D	–	–
Chebarkulskaya 3	50S	CQPKG/E	5S	CQPKG/E	60S	–	10S	CQPKG/E	20S	–	0	–
Eritrosperum 85-08	50S	THTTR/B	5MR	THTTR/B	0	–	10S	–	10S	–	0	–

Примечание. I — infestation, P/SSR — phenotype/SSR genotype S — response type 3-4 points, MR — response type 1-2 points, MS — response type X. Dashes in the table mean that samples were not analyzed..

observed (H_o) heterozygosity, fixation index (F) and Shannon's index (I). Genetic differences between the populations were determined by Nei D indices on M. Nei and F_{st}, which were calculated by AMOVA (GenAlEx) (for 999 permutations). Dendrogram of the genetic similarity in virulence and microsatellite loci among regional populations was constructed in the NTSYSpc 2.21 software for the Φ_{PT} and F_{st} indices. The data of microsatellite analysis and virulence assessment were compared using the Mantel test based on the distances between the relevant matrices for the virulence and SSR markers (for F_{st} and Nei D indices).

Results. Ecogeographic stude]y of wheat samples in the Western Siberia, the Urals and Kazakhstan are held annually according to the KASIB program to realize population researches of the causative agent of leaf rust on a vast territory (over 1000 km) and a single set of varieties, This excludes the selective pressure of the host plant which may interfere with the results of virulence analysis.

Using 20 Tc*Lr*-lines, 11 virulence phenotypes were detected (Table 1). Phenotypes identical in all locations were detected on varieties Duat (PQTKH, av.: Tc*Lr2a*, Tc*Lr2b*, Tc*Lr15*, Tc*Lr19*, Tc*Lr24*, Tc*Lr26*), Terzia (TQPTR, av.: Tc*Lr18*, Tc*Lr19*, Tc*Lr24*, Tc*Lr26*), Omskaya 35 (TGTTR, av.: Tc*Lr9*, Tc*Lr19*, Tc*Lr24*, Tc*Lr26*), Memory of Aziev (TGTTR), Saratovskaya 29 (TGTTR), Chebarkulskaya 3 (CQPKG, av.: Tc*Lr1*, Tc*Lr2a*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr11*, Tc*Lr15*, Tc*Lr19*, Tc*Lr20*, Tc*Lr24*, Tc*Lr26*) and on Eritrosperum 85-08 (THTTR, av.: Tc*Lr9*, Tc*Lr19*, Tc*Lr24*) line. Russian and North Kazakhstan isolates obtained from Astana 2, Lutescens 1003 (TGPTR, TGTTR) and Lutescens 6/04-4 (THPTR, THTTR) samples differed in virulence to Tc*Lr11,* and from ГВК 2074/4 (THTTM, THPTM) lines and Luteszens 715 (TGPTM, TGTTM) — to Tc*Lr18.* Karaganda isolates (TGPTR) from Stepnaya 53 variety differed from the Omsk and Pavlodar isolates (TGTTM) from the same varieties in virulence to Tc*Lr18* and avirulence to Tc*Lr11.*

More significant differences in the virulence spectrum between geographical populations were observed on line Lutescens 34/08-19 (PQTKH and TQTTR) and Rodnik variety (THTTR, MGTKH, TGTTR). These samples are less affected by pathogen (Lutescens 34/08-19 — from 0 to 20 %, Rodnik — from 10 to 50 %) then others studied (see Table 1). Despite some differences between the Russian and Kazakh populations, which were collected from several wheat samples, in general, we did not observe significant changes in the detected virulence spectrum of the pathogen. The phenotype detected in Chebarkulskaya variety 3 (CQPKG) has 10 alleles of virulence, that is less than in other wheat samples where the number of identified phenotypes is from 12 (MGTKH) to 17 (TNTTR).

Using PCR technique, genes *Lr26* (GVK 2074/4, Erythrosperum 85-08), *Lr9* and *Lr10* (Duet), *Lr9* and *Lr34* (Lutescens 34/08-19), *Lr26* and *Lr1* (Lutescens 6/04-4), *Lr10* and *Lr34* (Omskaya 35), *Lr*10 (Memory of Aziev, Saratovskaya 29, Stepnaya 53), *Lr1* and *Lr10* (Rodnik), *Lr9* (Tertsia, Chebarkulskaya 3) were identified in infectious samples. Genes *Lr1*, *Lr9*, *Lr10*, *Lr26* and *Lr34* are ineffective in Western Siberia, the Urals and Northern Kazakhstan conditions [4, 6, 12]. Meanwhile, their specific combinations can enhance field resistance of wheat [19], which was observed in this experiment for Lutescens line 34/08-19 with *Lr9* and *Lr34* genes (see Table 1).

Lr19 and *Lr24* genes showed high efficiency against Russian and Kazakh pathogen populations (Table 2), while *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr14a*, *Lr14b*, *Lr16*, *Lr17* and *Lr30* genes were widespread and absolutely ineffective.

In testing on Tc*Lr1*, Tc*Lr2a*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr9*, Tc*Lr15*, Tc*Lr18*,

Tc*Lr20*, Tc*Lr26* lines, the virulence frequency varied among isolates. Virulence to *Lr9* line was found in subpopulations collected on wheat samples with this gene (Duet, Tercia, Chebarkulskaya 3, Lutescens 34/08-19), to *Lr26* — in subpopulations from HVA lines 2074/4, Lutescens 6/04-4 and Erythrospermum 85-08 carrying *Lr26* gene. In general, the differences between regional populations in frequency of virulence to Tc*Lr9* and Tc*Lr26* depend on the presence of infectious material from the wheat plants carrying these genes. All isolates which were virulent to *Lr9* lines were avirulent to *Lr26* lines. This should suggests that the combination of *Lr9* and *Lr26* genes can provide effective protection of wheat plants against leaf rust, as observed for the genes combination *Lr19* + *Lr26*, *Lr19* + *Lr37* and *Lr19* + *Lr25* [20]. Avirulence to Tc*Lr20* was detected only in samples collected from Chebarkulskaya 3 variety. Certain differences between Russian and Kazakhstani populations were observed for virulence to Tc*Lr11*, i.e. the virulence was more often in case of *Lr11* in Russian populations.

2. Frequency (%) of *Puccinia triticina* Erikss. isolates virulent to Tc*Lr* wheat lines in the studied geographical populations (2016)

Thatcher line with *Lr*-gene	Russia			Kazakhstan		
	1	2	3	4	5	6
Tc*Lr19*. Tc*Lr24*	0	0	0	0	0	0
Tc*Lr1*	76.9	92.9	100	93.8	100	100
Tc*Lr2a*	46.2	85.7	80.0	93.8	87.5	100
Tc*Lr2b*	76.9	92.9	80.0	93.8	87.5	100
Tc*Lr2c*	76.9	92.9	100	93.8	87.5	100
Tc*Lr9*	23.8	14.3	40.0	18.8	12.5	0
Tc*Lr11*	76.9	92.9	80.0	50.0	50.0	62.5
Tc*Lr15*	46.2	85.7	80.0	93.8	87.5	100
Tc*Lr18*	100	78.6	100	100	87.5	100
Tc*Lr20*	76.9	92.9	100	93.8	100	100
Tc*Lr26*	46.2	21.4	0	12.5	25	25
Tc*Lr3a*. Tc*Lr3bg*. Tc*Lr3ka*. Tc*Lr14a*.Tc*Lr14b*. Tc*Lr16*.						
Tc*Lr17*. Tc*Lr30*	100	100	100	100	100	100
Number of isolates	13	28	6	16	16	12

Note. 1 — Chelabinskaya Province, 2 — Omskaya Province, 3 — Kurganskaya Province, 4 — Shortadinskaya Province, 5 — Pavlodarskaya Province, 6 — Karagandinskaya Province.

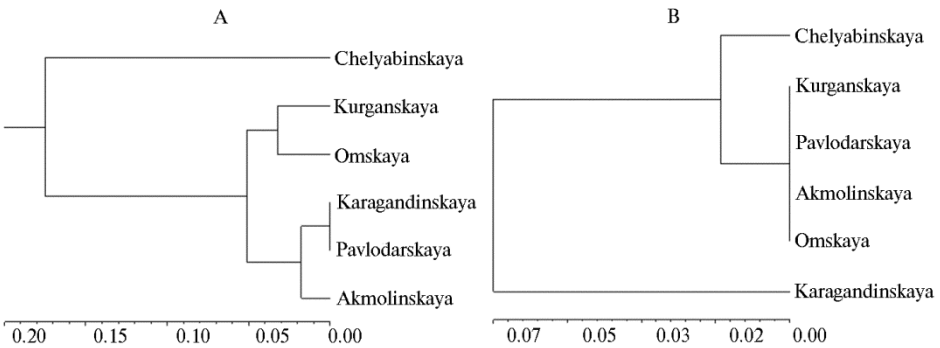

Fig. 1. UPGMA-dendrogram of genetic distances between *Puccinia triticina* Erikss populations for virulence (A) **and SSR markers** (B) (by F_{st}).

By the Nei D index, most of Russian and Kazakhstani populations are highly similar (Nei D = 0.02-0.7). The exceptions are Chelyabinsk and Karaganda populations (Nei D = 0.12). Index Φ_{PT} indicates a high similarity between Omskaya, Kurganskaya and Kazakhstanskaya populations. Chelyabinskaya is similar to Kurganskaya and differes from other studied populations (Fig. 1, A).

Analysis of polymorphism of 12 microsatellite loci detected 9 genotypes (4 in the Chelyabinskaya population, 4 in the Kurganskaya, 7 in the Omskaya, 4 in

the Pavlodarskaya, 5 in the Akmolinskaya, 2 in the Karagandinskaya) (see Table 1). Most of the genotypes belong to two and more populations, and only two are unique (one identified on Lutescens 715 line in Omskaya population, the other on Omskaya 35 variety in Kurganskaya population). A total of 21 polymorphic alleles are found. For most loci, two polymorphic alleles are identified, except for PtSSR55, PtSSR91 and PtSSR164 loci which are monomorphic (Fig. 2 at http://www.agrobiology.ru). We did not find alleles specific for a particular population. Note, we performed this study on ABI Prism 3500 genetic analyzer in which, unlike 4200 DNA Analyzer or 4300 DNA Analyzer (LI-COR, USA) used for similar purposes by other scientific groups [3, 12, 17, 18], sample preparation and analysis are fully automated that, with the same resolution, increases determination accuracy and data comparability.

Genetic diversity of *P. triticina* isolates on microsatellite loci are similar for all studied populations, except Karagandinskaya, which could be due to its low representation in the analysis (4 isolates). The mean number of alleles per locus (Na) varied from 1.2 in the Karagandinskaya population to 1.6-1.7 in the other studied, the number of effective alleles (Ne) was from 1.1 to 1.3-1.4, respectively. The observed heterozygosity (H_o) was higher than expected (H_e) for the Chelyabinskaya, Kurganskaya, Pavlodarskaya and Omskaya populations, which was confirmed by negative values of the fixation index (F). Values of H_o and H_e for Karagandinskaya and Akmolinskaya populations were similar (0.06 and 0.07; 0.16 and 0.19, respectively). Shannon index (I) was identical for all populations (0.3), except Karagandinskaya (0.1).

According to F_{st} index of genetic variations, the most of the studied populations, except for Chelyabinskaya and Karagandinskaya, do not differ for SSR markers ($F_{st} = 0.21$, $P = 0.003$) (see Fig. 1, B). We also obtained similar results by Nei D parameter (0.01-0.03 and 0.07, respectively). The identified differentiation between the Chelyabinskaya and Karagandinskaya populations can be explained by the contrast of wheat varieties from these two regions for *Lr*-genes, geographical remoteness (over 1000 km) of the populations and regional differences in climatic conditions. In the Karaganda region, leaf rust is noted sporadically in years favorable for the development of the pathogen. In the Chelyabinsk region, the disease occurs almost every year and belongs to a potentially dangerous group.

In whole, in comparing Russian and Kazakhstani populations, the indices of genetic distances indicate a high similarity (Nei $D = 0.007$, $F_{st} = 0.003$, $P = 0.31$), which agrees with the results of the earlier analysis of virulence [10, 20]. The low value of genetic divergence index (F_{st}) suggests the existence of a gene flow between the studied populations.

For all the populations except for Chelyabinskaya, the differentiation by virulence and microsatellite loci is quite close in pattern, i.e. by Mantel test for Nei D $r = 0.88$ ($P < 0.001$), for F_{st} $r = 0.43$ ($P < 0.001$). The similarity of Chelyabinskaya population with Akmolinskaya and Pavlodaraskaya populations by microsatellite loci is significantly higher than by virulence.

The tight connections among the West Asian Russian and North Kazakhstan populations indicate the existence of a single fungus population in these regions. Such results are in line with the reports of other researchers and the data obtained earlier [4, 10, 12, 21]. There are several assumptions on the emergence of the infection in the west of Asian Russia and Kazakhstan. The Ural Mountains are a geographical obstacle to spores spread from Europe to Asia, and the local direction of air flows is a physical obstacle [21]. It is shown [22] that the probability of spore migration from the North Caucasus territory to Kazakhstan is extremely

small, since the cyclone operating between the Caspian and Aral Seas and the anticyclone running down from the north along Western Siberia hinder the penetration of air mass. The diseases epiphytoty in Western Siberia and the Urals vs. its depression in the North Caucasus [21, 22], as well as the absence of isolates with virulence to *Lr9* in the North Caucasian populations [23] vs. their high frequency in Asian can be as a confirmation.

According to the opinion of other researchers, the epiphytotic development of leaf rust in Western Siberia and Northern Kazakhstan occurs only due to infection from the southern or southwestern regions of the European Russia [24]. The pathogen transfer from the sowings of the Middle Volga and western regions of Kazakhstan is not excluded [24, 25]. Thus, additionally to the exogenous infection in Western Siberia, there are its own sources independent of European ones [21, 22, 24]. Vegetation of the intermediate host plant *Isopyrum fumarioides* in the region can contribute to a full cycle of the pathogen development. It is reported [26] about the higher plasticity of the *P. triticina fungus* on *I. fumarioides* species**,** compared to the form affecting *Thalictrum* spp., which increases the competitiveness of local isolates. Affected crops of winter and spring wheat in the west of the republic and in neighboring regions of Russia can be the source of infection for Northern Kazakhstan. Favorable conditions for air infection transfer are created due to the prevalence of western winds during the growing season [27]. According to other data [12, 28], the contamination of spring wheat in Northern Kazakhstan occurs during the spore transfer from cultivated winter wheat by air from the southern part of the republic due to the lack of geographic barriers between the north and south of Kazakhstan.

Thus, using the approach which reduces the influence of the host plant on the estimates of virulence of the leaf rust causative agent, we have shown the high similarity of *Puccinia triticina* Erikss. populations in the Urals, Western Siberia and northern Kazakhstan. These data prove the assumption about existence of a single pathogen population in these regions. To prevent epiphytoty of *Puccinia triticina* on the adjacent territories of Russia and Kazakhstan, it is recommended to update constantly the stock of wheat varieties, to improve genetic diversity, to use mosaic sowings of varieties and optimal areas occupied by genetically homogeneous varieties.

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ANTIFUNGAL ACTIVITY AGAINST PATHOGENS OF CEREALS AND CHARACTERIZATION OF ANTIBIOTICS OF *Streptomyces* sp. STRAIN K-541 ISOLATED FROM EXTREME ECOSYSTEMS IN KAZAKHSTAN

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Abstract

The general requirement for biologicals is that they must be insensitive to climate change and soil conditions, including soil physicochemical composition, fertility levels, and pH values. Actinomycetes isolated from extreme habitats are able to produce biologically active substances not only under neutral conditions but also in saline, alkaline and acidic environments, which determines their importance in the biopreparations, being developed for plant protection. This study is the first to report the *Streptomyces* sp. strain K-541 antibiosis against the causative agents of several cereal fungal infections under various environmental conditions and the identification of the antibiotic produced. *Streptomyces* sp. strain K-541 isolated from extreme ecosystems of Kazakhstan was cultured under neutral (pH 7.0) and alternative growth conditions at 25.0 g/l NaCl (pH 7.2) or 2.5 g/l Na_2CO_3 (pH 8.0). Antifungal activity was determined in agar block diffusion experiments and under paired co-incubation with phytopathogenic fungi *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. heterosporum* Nees, *F. sporotrichiella* Sherb., *Piricularia oryzae* Cavara, *Alternaria triticina* Prasada & Prabhu, *A. alternate* (Fr.) Keissl., *Bipolaris sorokiniana* (Sacc.) Shoemaker, and *Aspergillus niger* van Tieghem. For antibiotic A-541 production, the strain was cultured on an orbital shaker (180-200 rpm) for 120 hours at 28 °C. The antibiotic was extracted with organic solvents and analyzed using thin layer chromatography and spectrophotometry. The studies have shown high antifungal activity of K-541 against all the phytopathogens examined. After 72 hour incubation at 25 °C the growth inhibition zones were 20-56 mm in diameter depending on growth conditions which simulated different ecological niches. In co-culturing the strain K-541 and the phytopathogenic fungi, the fungal colonies decreased 1.8-2.7 times in diameter indicating the possibility of K-541 introduction into soil biocenoses for biocontrol of cereal fungal pathogens. High inhibition of growth was also observed under saline (2 % NaCl) and alkaline (0.2 % Na_2CO_3) conditions. The antibiotic produced by strain K-541 was classified as a member of polyene group, a subgroup of the hexaenes. So strain K-541 is recognized as promising for the development of a new biopreparation with fungicidal activity against causal agents of cereal fungal infections under different environmental conditions.

Keywords: extremophilic streptomycete, antibiotic, antifungal activity, phytopathogenic fungi, wilt, rice blast disease, leaf blight, common root rot, spot blotch, mold, *Fusarium solani*, *F. oxysporum*, *F. heterosporum*, *F. sporotrichiella*, *Piricularia oryzae*, *Alternaria triticina*, *A. alternata*, *Bipolaris sorokiniana*, *Aspergillus niger*, cereal crops.

Recently, the spread of toxinogenic fungi, the causative agents of cereal crop diseases, has increased dramatically. A loss of more than 40 % grain yield results from attack of *Fusarium*, *Alternaria*, *Pyricularia* and *Aspergillus* species [1]. When infecting plants, micromycetes, along with a significant economic damage to agriculture, are environmentally harmful. However, the widespread use of chemical agents for protecting plants against fungal diseases also poses a particular danger, since the negative influence of fungicides increases with time. The

situation is worsening by the development of fungal resistance to fungicides, followed by the need to increase the dose of chemicals. In integrated plant protection, biological methods are relevant. This approach particularly includes the use of microorganisms and their secondary metabolites to create effective, environmentally friendly biofungicides with different mechanisms of action, as well as to increase plant resistance to abiotic stresses. The discovery of microorganisms forming compounds with a wide range of biological activity is of current interest, for the search for which various ecosystems are of interest, including the poorly studied extremes [2-4].

Actinomycetes are producers of secondary metabolites with antibacterial, antifungal, insecticidal, and other actions (5-7). They are an important component of microbiocenoses, and their quantitative and qualitative composition is a factor characterizing the ecological state of natural ecosystems [8]. Many types of actinomycetes, including those belonging to the genus Streptomyces, are known as antimycotic agents that inhibit pathogenic fungi [9-11]. The ability of actinomycetes to colonize the surface of plant roots, to produce antibiotics and extracellular enzymes ensures their high efficiency as biocontrol tools of plant protection against diseases [12-14].

Grain farming is the main branch of plant growing in Kazakhstan, where saline land accounts for 15.2 % of the total area of rural land. The remaining soils are also saline to varying degrees, heterogeneous in composition with low humus content [15]. Biological products for use in such difficult conditions should use microorganisms that retain biological activity in various ecological niches. Salinization of the soil and pH interfere with the protective and stimulating effect of microorganisms. Actinomycetes from extreme habitats can grow and produce biologically active substances during salinization, in alkaline and acidic environments [16, 17]. Despite the abundance of information on the biological properties of actinomycetes, their ability to regulate growth of pathogenic microorganisms, including phytopathogenic fungi, under strong salinity, high and low pH conditions has been little studied [18, 19].

In this paper, we for the first time used the biotechnological potential of extremophilic actinomycetes to develop a universal antifungal biologicals.

Our subjective was to study the antagonistic properties of the strain *Streptomyces* sp. K-541 to fungal pathogens of grain crops in different environmental conditions and to identify groups of the obtained antibiotics.

Techniques. Strain *Streptomyces* sp. K-541 isolated from an extreme ecosystem (saline solonchak, Kostanay region, Republic of Kazakhsta) was grown on three glucose-yeast nutrient media containing (g/l) glucose (2.0), yeast extract (1.0), peptone (2.0), agar (20.0), pH 7.2 (growth medium 1); glucose (2.0), yeast extract (1.0), peptone (2.0), NaCl (25.0), agar (20.0), pH 7.2 (growth medium 2); glucose (2.0), yeast extract (1.0), peptone (2.0), Na2CO3 (2.5), agar (20.0), pH 8.0 (growth medium 3). The pH value was adjusted with 0.1 N NaOH solution using a MP220 pH meter (Mettler-Toledo International, Inc., USA).

Strain K-541 was grown on three variants of agar and cultured for 7 days at 28 °C. Antifungal properties were determined by agar block technique [20]. Antifungal activity of the strain grown under neutral, saline and alkaline conditions was evaluated in tests with five genera of phytopathogenic fungi which are the causative agents of the main fungal diseases of wheat and rice. These are *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. heterosporum* Nees, *F. sporotrichiella* Sherb.; *Piricularia oryzae* Cavara; *Alternaria triticina* Prasada & Prabhu, *A. alternate* (Fr.) Keissl.; *Bipolaris sorokiniana* (Sacc.) Shoemaker; *Aspergillus niger* van Tieghem. Czapek-Dox agar melted and cooled to 40-50 °C was

inoculated with a suspension of conidia of phytopathogenic fungi (108 CFU/ml, 1 ml per 100 ml medium) and poured into Petri dishes. Blocks of the grown culture were cut out with a drill (7 mm in diameter), transferred to Petri dishes pre-inoculated with test cultures of phytopathogenic fungi, and allowed for 72 hours at 25 °C. Blocks cut from pure agar media served as controls. Antagonistic activity was estimated by the lysis zone diameter of the test cultures measured with 1 mm accuracy.

For spores, strain K-541 was grown on Gause mineral salts agar 1 for 7 days at 28 °C. Spores were removed by washing. Spores (10^9 CFU/ml) were inoculated in liquid nutrient medium with oatmeal (1 ml per 100 ml). The growth medium contained (g/l) glucose (15.0), oat flour (15.0), CaCO3 (2.5), and NaCl (5.0); pH 7.0-7.2.

For antibiotics, strain K-541 was cultured in 750 ml Erlenmeyer flasks with 100 ml oat medium (a circular shaker, 180-200 rpm for 120 hours at 28 °C). Bacterial biomass was pressed to 70 % humidity, weighed and extracted with 96 % ethanol (3 ml per 1 g biomass). Antibiotic A-541 was extracted using a mechanical stirrer (RW 20 digital, IKA, Germany) for 2 hours at room temperature in a laboratory fume hood, then for 3 hours in a refrigerator. The solvent was removed under vacuum (a rotary evaporator RV 10 Basic, IKA, Germany) at 35-40 °C. The antibiotic was extracted from the culture fluid by n-butanol (pH 7.0). Extracts were fractionated on a separating funnel, evaporated under vacuum and re-extracted by ethyl alcohol.

Ethanol extracts from K-541 strain culture fluid and biomass were chromatographed on Silufol plates R, UV 254 (Cavalier, Czech Republic), Sorbfil (Sorbpolymer, Russia) and DC-Alufolien Kieselgel 60 (Merck, USA) with chloroform + ethanol (20:1, 20:7), n-butanol + acetic acid + water (2:1:1, 3:1:1), chloroform + ethyl acetate (1:1), ethanol + butanol + water (4:1:1).

Antibiotic was detected visually on chromatograms by luminescence in UV light (a UFS-254/365 irradiator, ZAO Tekhnok Scientific and Production Association, Russia) and bioavtographically with *Fusarium oxysporum* as a test organism. Zones corresponding to the position of the individual components in the chromatogram were cut out and placed on the agar with the test organism. The active zones were removed from the plates, eluted with ethanol, filtered, and the extract was evaporated. The UV and visible absorption spectra of the total preparation and its individual fractions were measured in 96 % ethanol (a Cary 60 UV-Vis spectrophotometer, Agilent Technologies, USA).

Statistical processing was performed as per V.Yu. Urbach [21]. The tables show the mean values *(M)* and standard errors of the means (±SEM).

Results. It was shown that *Streptomyces* sp. K-541 has high fungicidal effect against all studied phytopathogenic fungi (Table 1), with 30-45 mm zone when grown at neutral conditions, 40-48 mm zone at salinity, 20-33 mm at alkaline conditions against *Fusarium*; 32-35, 36-37, 20-22 mm, respectively, against *Alternaria*, 36-38, 38-40, 30 mm against *Bipolaris*, and 40, 56, and 50 mm against *Piricularia*. It should be noted that streptomycetes are widely distributed in soils of various types, and for acidic soils, species that can suppress the development of phytopathogenic fungi at low pH are also described [22, 23].

To study possible use of extremophilic K-541 strain as a biocontrol agent of fungal infections, when introdused into the soil biocenoses, the strain was cultured with phytopathogenic fungi on Czapek-Dox (Table 2, Fig.). The colonies of *Fusarium oxysporum* was 2.2-2.1 times less in diameter, of *Piricularia oryzae* — 1.8-2.7 times less, of *Alternaria alternata* — 2.2-2.4 times less, of *Bipolaris sorokiniana* — 2.0-2.2 times less, and of *Aspergillus niger* — 2.4-2.5 times less. These

data indicate a significant inhibition of fungal growth not only at normal condition but also under salinity (2.0 % NaCl, medium B) and at an alkaline pH (0.2 % Na_2CO_3, medium C).

1. Antifungal activity of *Streptomyces* sp. K-541 against causative agents of the main fungal diseases of wheat and rice, depending on the culture medium (*M*±SEM)

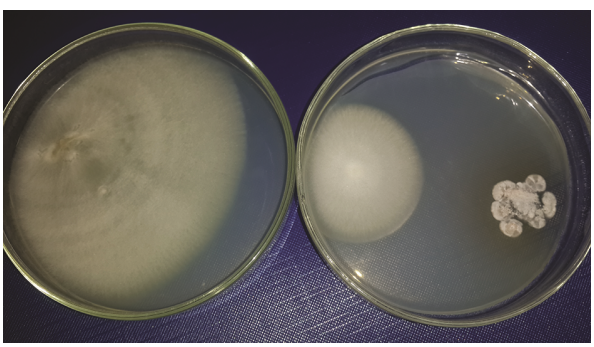
Fungal species	Lysis zone, mm		
	medium 1	medium 2	medium 3
Fusarium oxysporum	31±0.3	42±0.2	20±0.2
F. heterosporum	40±0.3	45±0.1	28±0.4
F. solani	45±0.2	48±0.4	33±0.2
F. sporotrichiella	40±0.1	45±0.1	32±0.3
Aspergillus niger	46±0.3	50±0.3	30±0.4
Piricularia oryzae	40±0.5	56±0.1	50±0.6
Alternaria alternata	32±0.3	36±0.4	20±0.1
A. triticina	35±0.3	37±0.2	22±0.3
Bipolaris sorokiniana	38±0.1	40±0.5	30±0.6
Control (no test culture)	0	0	0

N o t e. Glucose-yeast agar growth media 1-3 are described in the section *Techniques*.

2. Antifungal effect of Streptomyces sp. K-541 in co-culture with fungal phytopathoges of wheat and rice (*M*±SEM)

Fungal species	Variant	Diameter of colonies, mm		
		medium A	medium B	medium C
Fusarium oxysporum	Control	116±0.3	112±0.2	100±0.2
	Test	52±0.1	50±0.3	48±0.2
Piricularia oryzae	Control	64±0.2	66±0.3	60±0.3
	Test	36±0.1	30±0.1	22±0.1
Alternaria alternata	Control	88±0.4	88±0.5	86±0.3
	Test	40±0.2	38±0.2	36±0.2
Bipolaris sorokiniana	Control	48±0.1	44±0.4	42±0.4
	Test	22±0.3	22±0.1	20±0.1
Aspergillus niger	Control	114±0.3	114±0.2	112±0.5
	Test	48±0.5	46±0.1	44±0.3

N o t e. Medium A is Czapek-Dox agar, medium B is Czapek-Dox agar with 2 % NaCl, and medium C is Czapek-Dox agar with 0.2 % Na_2CO_3, pH 8.0.



Inhibition of *Fusarium oxysporum* growth in co-culture with *Streptomyces* sp. K-541: on the left — control (day 10 of growth; Czapek-Dox medium, 2 % NaCl, pH 7.0).

Thin-layer chromatography indicates that antibiotic A-541 is a complex mix. The A-541-1 extracted from the culture fluid and the A-541-2 from the biomass are identic in composition. The best separation results from use of n-butanol:acetic acid:water (3:1:1) system which produces 8 individual chemical compounds with UV luminescence. This system was further used for the preparative isolation of antibiotically active fractions on silica gel plates.

Bioavtography with *Fusarium oxysporum* as a test organism showed that only components IV with Rf = 52 (antibiotic A-541-1) and Rf = 0.46 (antibiotic A-541-2) have a bioactivity in the n-butanol:acetic acid:water (3: 1: 1) extracts. The bioactive IV component produces a homogeneous band under thin-layer chromatography patterns with n-butanol:acetic acid:water (2:1:1, 3:1:1). For A-541-2, the active component was isolated from the zone with Rf = 0.46. The absorption spectra of the antibiotic complex A-541 and component IV are identical and have the main UV peaks at λ 317, 330, 354 and 376 nm. In the UV

spectrum, there are peaks characteristic of polyene antibiotics which are closest to those in the hexaen subgroup (at при 330, 354, 376 nm) [24].

Thus, the strain Streptomyces sp. K-541 is a promising agent against harmful fungal infections of grain crops (wheat and rice) due to its high antifungal activity against all studied phytopathogens. Streptomyces sp. K-541 can be introduced into soil biocenoses for biocontrol of fusarium pathogens of cereal crops in neutral and alkaline soil conditions and under salinization. This is especially valuable for crop production in Kazakhstan where the soil is heterogeneous in composition and is characterized by high salinity. The isolated antibiotic A-541 is preliminarily defined as a polyene, particularly a hexaene-type antibiotic.

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SELECTION OF SPRING SOFT WHEAT (*Triticum aestivum* L.) VARIETIES FOR THE ADAPTABILITY IN THE CONDITIONS OF STEPPE ZONE OF THE AKMOLINSK REGION, KAZAKHSTAN

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Abstract

The spring soft wheat (*Triticum aestivum* L.) planting acreage makes 78 % of the crops of cereals grown in Akmolinsk region, the North Kazakhstan. The wheat productivity can reach 1.4-1.8 t/ha which makes it possible to harvest high yield with high grain quality and high protein level. That is important when cultivating strong wheats, the so called improvers. At the present time, searching for the reserves to increase yield and to improve grain quality in wheats is of interest. Here, the key role is given to a variety because over 20 % of an increase in the crop yield is due to varietal features. This article presents data on phenology, nodal root formation, the main yield elements, and the total yield of spring wheat (*Triticum aestivum* L.) promising varieties Karagandinskaya 22, Karagandinskaya 29, Karagandinskaya 70, Seke, Sary-Arka, and Stepnaya 60. The estimations have been carried out according to the Kazakhstan State Crop Variety Testing protocol. Field trials were carried out in 2011-2013 using fallow land as a predecessor in the test field of Ualikhanov Kokshetau state University (Kokshetau, Kzakhstan). The conditions of the steppe zone were favorable for nodal root development which number per plant reached 5.5 for Karagandinskaya 22, 4.5 for Stepnaya 60, and 5.2 for Seke. The number of nodal roots during plant growth increased from tillering to full grain ripeness. In the arid conditions of the steppe zone the length of growing season is of importance. Among the varieties, the vegetation period was optimal in Karaganda 22 (94 days), with an average 96 day period for all the varieties studied. The main components of wheat yield were shown to be beneficially influenced by the climate conditions. The plant number per 1 m^2 at harvesting was 230-282, the productive tiller number per 1 m2 was 201-333, the seed number per ear was 20-30, and the 1000 seed weight was 36.4-49.6. Grain productivity of the varieties Karagandinskaya 22, Sary-Arka, and Stepnaya 60 was 4.0, 3.6, and 3.4 t/ha, respectively. So their yielding was higher compared to only 1.8 t/ha for the standard variety Svetlanka. During selection for adaptability in the conditions of steppe zone of the region, the main emphasis should be given to the number of kernels per ear (r = 0.86±0.13) and the 1000 grain weight (r = 0.76±0.12). In a model variety for the steppe zone of the region, the main yielding parameters must be as follows: 96 day-length of vegetation, 25 to 30 seeds per ear, productive tillering of 1.1 to 1.2, and the yield of 3.0-4.0 t/ha. The 1000 seed weight should be 33-36 g for the fraction of mid-size seeds, and more than 38-45 g for larger seeds.

Keywords: spring soft wheat, sort, nodal roots, yielding capacity, elements of the structure

Spring soft wheat is the leading cereal in the northern regions of the Republic of Kazakhstan, where its acreage, according to the latest data, is 13.5 million ha. Cultivated varieties stand out for the high quality of their grain, which is not inferior to international standards. However, due to extremely changeable weather conditions, grain yield varies greatly over the years (from 0.4 to 2.7 t/ha), which indicates a reduced biological productivity of the climate [1].

Varieties are the basis of the production of any crop products. They

largely determine the zonal technologies of cultivation, size, quality, energy efficiency of the products obtained (2). In modern economic and climatic conditions, the main requirements for varieties are high yields, the possibility of cultivation using energy-saving technologies, environmental resistance to stresses, plasticity, high grain quality, and resistance to damage by pathogens and pests [3, 4]. A prerequisite for the successful creation of such varieties may be a different response of plants to changes in environmental conditions, which is under genetic control [5]. Namely, the drought resistance of a variety depends on the genomes of the parents, especially if one of them belongs to the wild-growing related species [6, 7].

The adaptive capacity helps a variety to uncover its potential yield [8]. In terms of fertility of soils, their optimum moisture content and daylight length, the region of Northern Kazakhstan is suitable for the cultivation of spring soft wheat. These conditions make it possible to grow high-yielding high-protein varieties. The intrazonal identity of varieties affects the yield structure [8]. The ability to adapt offers the varieties an advantage in increasing the number of nodal roots, the coefficient of productive tilling capacity, grain content and yield. Namely, the varieties of the Karaganda selection showed good results with respect to the above indicators in the Akmola Region.

In this study, for the first time, we identified the main features and parameters of the mid-ripening spring soft wheat variety model for the steppe zone of Akmola Region in terms of the duration of the interstage and vegetation periods, the number of nodal roots and the main elements of the yield structure.

The objective of the work was to isolate varieties of spring soft wheat (*Triticum aestivum* L.) according to a set of features maximally adapted to the conditions of Northern Kazakhstan, as well as to assess the relationship between the elements of the productivity structure in mid-ripening varieties and the final grain yield.

Techniques. The experiment was carried out using fallow land as a predecessor in the test field of Sh. Ualikhanov Kokshetau State University (Republic of Kazakhstan) in 2011-2013. The experiment area was 3 m^2, with the total area of 5 m^2. Repetition was 4-fold. Variants were placed randomly. Sowing was carried out at the optimum time (May 18-20) manually with a seeding rate of 350 viable kernels per 1 m^2. The soil of the experimental plot is ordinary chernozem with humus content of 3.6 %, pH 6.0-6.5.

The material was promising varieties of spring soft wheat (*Triticum aestivum* L.) of the mid-ripening group created in the Karaganda Research Institute of Crop Science and Plant Breeding: Karagandinskaya 22, Karagan-dinskaya 29, Karagandinskaya 70, Seke, Sary-Arka, and Stepnaya 60. The variety Svetlanka registered in the Akmola Region was used as a standard. We evaluated the duration of interstage and vegetation periods, the dynamics of the nodal roots, the yield and the main elements of its structure: the number of plants, the number of productive stems, the productive tilling capacity, the number of kernels per ear, and the thousand-kernel weight.

The experimental data were analyzed statistically in Microsoft Excel 2010. When determining the means (M), their standard errors (±SEM) were calculated. We calculated the least significant difference ($LSD_{0.05}$), as well as coefficients of variation (*Cv*, %) and correlation (*r*) according to B.A. Dospekhov [9].

Results. The impact of agrometeorological conditions on the yield of spring wheat varieties in the years of the study was not the same. Namely, the hydrothermal coefficient (HTC) in 2011 and 2012 (1.2 and 1.1, respectively) was close to the long-term average annual indicator (0.9) in the zone, while in 2013, which turned out to be overmoistened, the HTC was 2.9.

The soft wheat endures the negative impact of extreme conditions of the spring-early summer drought better during the tillering period, but its productivity is greatly reduced if the impact falls on the booting stage, when ear initiation and differentiation occur (organogenesis stages V–VII). Under the conditions of the steppe zone of Northern Kazakhstan, where droughts are constantly observed, wheat varieties with an extended tillering period are more adapted to local conditions [11, 12]. Yield formation depends on the length of the first half of the vegetation period [13].

During the study years, the average length of the vegetation period for mid-ripening varieties was 98.28 ± 1.88 days (Table 1). Due to the prevailing climatic conditions, the interstage seedling-heading period lasted 41 days. The lack of precipitation in the first half of the summer accelerated the passage of this period. Lengthening of the heading-ripening period (50-57 days) was associated with a decrease in temperature in July and heavy rains in the second decade of August. Among the studied varieties, Karagandinskaya 22 had the shortest (94 days) vegetation period (Table 1). The vegetation period lengthens in case of excessive moisture [13]. The HTC during the observation period was excessive (an average of 3 years — 1.7).

1. Duration (in days) **of interstage periods and vegetation in mid-ripening spring soft wheat varieties (*Triticum aestivum* L.)** (Republic of Kazakhstan, 2011-2013)

Variety	Seedling—tillering	Tillering—booting	Booting—heading	Heading—ripeness	Vegetation period
Svetlanka (standard)	12	14	16	57	99
Karagandinskaya 70	13	14	16	56	99
Seke	13	15	16	55	99
Karagandinskaya 22	14	15	15	50	94
Stepnaya 60	14	14	14	57	99
Karagandinskaya 29	14	14	15	56	99
Sary-Arka	14	14	15	56	99
$M \pm$ SEM	13.42±0.48	14.28±0.48	15.28±0.75	55.28±2.42	98.28±1.88
$LSD_{0.05}$	0.08	0.07	0.07	0.14	0.20

The average coefficient of variation over 3 years for the seedling–heading period was 16.1%, for heading–waxy ripeness 21.4%, for the entire vegetation period 9.2%, and depended on the weather conditions and genetic characteristics of the varieties. The results of our studies showed a fairly high positive correlation between the length of the vegetation period and the yield of spring soft wheat ($r = 0.83 \pm 0.18$). Previously, R.K. Kadikov et al. [14] concluded that during a moist year with moderate temperature, the mid-ripening variety shows high grain yield.

The degree of supply of the aboveground organs with water and soil nutrition elements depend on the nature, growth and development, distribution and other morphostructural characteristics of the root system, which affects the stability and productivity of plants [15]. In terms of the number of nodal roots, we identified varietal differences, the manifestation of which is significantly influenced by both environmental conditions and the genetic characteristics of plants. Similar conclusions were drawn by other authors using the example of the Sonora and Currawa genotypes [16, 17]. In the work by A.M. Manschadi et al. [18], the arid variety Seri M 82 compared to the standard Hartog had a compact root system.

In the conditions of a frequent spring-summer drought, the time of nodal root initiation and growth and their late dying-off at the end of the vegetation become important. Our studies of mid-ripening varieties showed that the initiation of the tillering node for most of them began during the fourth leaf period. The works of foreign authors contain information that the nodal roots can be

distinguished during the third leaf period, since they are expanded in diameter and are formed from the tillering node from a different angle [18]. In spring soft wheat varieties during the tillering stage one plant had from 1.9 (Karagandinskaya 29) to 3.3 nodal roots (Karagandinskaya 22), while the standard Svetlanka had 2.6 nodal roots. The average number of nodal roots was (2.78 ± 0.45) per plant (Table 2). According to M. Watt [19], spring wheat was observed to have one nodal root, rarely two. Therefore, agricultural practices aimed at the accumulation and preservation of moisture in the upper layers of the soil will contribute to the better development of nodal roots [8].

The growth and development of the root system, like other morphophysiological characteristics, always vary [8]. In wheat, in terms of the share of influence on grain yield, such a characteristic as the nodal root formation accounts for 50% [20]. During the booting stage, we observed the highest growth in the nodal roots (3.9) in plants of the Karagandinskaya 22 variety, while Karagandinskaya 29 had the smallest value of this indicator (2.5). The average value of this characteristic in all varieties was (3.27 ± 0.42) roots per plant. During the heading period, the largest number of nodal roots was observed in the Karagandinskaya 22 and Seke varieties, i.e. 4.6 and 4.1 roots per plant, respectively; the average value was 3.75 ± 0.45 roots per plant. During the milky ripeness stage, the Seke and Karagandinskaya 22 varieties were characterized by the best indicators, i.e. 4.9 and 5.2 roots per plant, with the average value of (4.58 ± 0.44) roots per plant.

According to V.K. Movchan (quoted in 20), the varieties capable of forming a large number of seminal roots (5 or more) have a relatively high drought resistance, produce stable yields and can be used to create new productive genotypes.

2. Number of nodal roots (roots per plant) **in mid-ripening spring soft wheat varieties (*Triticum aestivum* L.) depending on the development stage** (Republic of Kazakhstan, 2011-2013)

Variety	Tillering	Booting	Heading	Flowering	Milky ripeness	Waxy ripeness	Complete ripeness
Svetlanka (standard)	2.6	3.3	3.6	4.1	4.7	5.0	5.1
Karagandinskaya 70	2.7	3.4	3.6	4.5	4.7	4.9	5.0
Seke	3.1	3.2	4.1	4.6	4.9	5.1	5.2
Karagandinskaya 22	3.3	3.9	4.6	4.8	5.2	5.3	5.5
Stepnaya 60	3.0	3.5	3.6	3.9	4.3	4.4	4.5
Karagandinskaya 29	1.9	2.5	3.2	3.6	3.8	3.9	3.9
Sary-Arka	2.9	3.1	3.6	4.1	4.5	4.7	4.9
$M \pm$ SEM	2.78±0.45	3.27±0.42	3.75±0.45	4.22±0.42	4.58±0.44	4.75±0.47	4.87±0.52
$LSD_{0.05}$	0.05	0.03	0.03	0.05	0.05	0.09	0.04

The variability in the number of nodal roots of the varieties prior to the booting stage was small (*Cv* 2.2-5.1%). This indicator increased towards the heading stage (8.1%), and then slightly decreased during the kernel milky ripeness period (7.2%). In terms of the number of nodal roots, the correlation relationship with the yield of the spring soft wheat varieties studied was moderately positive ($r = 0.62 \pm 0.20$). It should be noted that most of the varieties were characterized by the early and simultaneous emergence of nodal roots, the formation of which continued until the end of the vegetation period, which was associated with rainfall and adequate moisture in the soil during the years of observation. A number of ecological and morphophysiological parameters of the root system can be used as additional criteria for predicting the productivity of a genotype under specific conditions [8].

The development of the root system enhances the integration of elements of the productivity structure in promising varieties [21]. Of the seven varieties we studied, Karagandinskaya 22 and Seke stood out for their productive

tilling capacity (1.2) surpassing the standard (1.0). According to Ye.A. Korenyuk, the productive tilling capacity account for 94.1 to 96.8% [22] in the spring wheat yield structure. According to Yu.S. Krasnova, the yield of spring soft wheat grain depends on the high value of productive tilling capacity ($r = 0.4 \pm 0.06$). This feature should be included in breeding programs to achieve a stable grain yield [14].

In addition, the Karagandinskaya 22, Sary-Arka, Stepnaya 60 varieties were distinguished by a higher thousand-kernel weight, which directly depended on the temperature conditions and the amount of precipitation. A similar effect was noted in field tests during the study of grain quality indicators in spring wheat varieties with *Lr*-translocations depending on temperature and humidity conditions (23). In our experience, the coefficient of variation of the thousand-kernel weight was 13.2%, and the conjugate dependence was expressed by direct correlation with yield ($r = 0.76 \pm 0.12$). The thousand-kernel weight varied from 36.4 g in the Karagandinskaya 70 variety to 49.6 g in the Karagandinskaya 22 variety and averaged (42.00 ± 5.06) g.

A similar pattern, reflecting the high coefficient of heritability of the number of seminal roots, which closely correlates with a change in the thousand-kernel weight and ear grain content, was noted earlier when studying the manifestations of drought resistance in spring soft wheat in the Central Kazakhstan [15].

3. Элементы структуры урожая у среднеспелых сортов яровой мягкой пшеницы (*Triticum aestivum* L.) в зависимости от фазы развития (Республика Казахстан, 2011-2013 годы)

Variety	Number, pcs./m^2		Productive tilling capacity	Number of kernels per ear, pcs.	Thousand-kernel weight, g	Biological yield, t/ha
	of plants	of productive stems				
Svetlanka (standard)	277	290	1.0	20	39.6	1.8
Karagandinskaya 70	297	201	0.6	27	36.4	2.9
Seke	230	288	1.2	27	40.2	2.5
Karagandinskaya 22	270	333	1.2	25	49.6	4.0
Stepnaya 60	282	269	0.9	28	43.0	3.4
Karagandinskaya 29	260	280	1.0	28	37.4	2.7
Sary-Arka	233	236	1.0	33	47.8	3.7
$M \pm$ SEM	264.14±25.00	271.00±42.23	0.98±0.20	26.85±3.89	42.00±5.06	3.00±0.75
$LSD_{0.05}$	13.00	25.70	0.10	6.97	5.75	0.45

The number of kernels in an ear depends on weather conditions during flowering. The increase in the amount of grain is compensated by a significant decrease in its weight (up to 5%) [24, 25], but we obtained different results during our study. Namely, in case of Sary-Arka, as the number of kernels per ear increased (33 pcs.), the thousand kernel weight increased (47.8 g) and, accordingly, the biological yield increased (3.7 t/ha) (Table 3). The coefficient of variation of the number of kernels per ear was 23.3%, the correlation dependence showed a close relationship between the ear grain content and yield ($r = 0.86 \pm 0.13$). However, in case of the Karagandinskaya 22 variety with the ear grain content of 25, the thousand-kernel weight was 49.6 g, and yield 4.0 t/ha. Therefore, when selecting varieties, the focus should be on the absolute grain weight (thousand-kernel weight) (40-43 g) in combination with such a quantitative feature of an ear as high grain content (up to 27 pcs.) [26, 27]. In terms of ear grain content, the most adapted selection varieties were Sary-Arka (33 pcs.), Karagandinskaya 29 and Stepnaya 60 (28 pcs.).

Varieties should be placed on high agricultural backgrounds, which will allow them to form high yields thanks to their responsiveness to changing environmental conditions [28]. With an increase in yield of up to 1.5 t/ha in the Republic of Kazakhstan, it is possible to harvest up to 20-22 million tons of grain

annually. The use of the Karagandinskaya varieties of spring soft wheat in the Akmola Region in rapidly changing weather conditions in a sharply continental climate contributes to an increase in the gross grain harvest [29]. Among promising varieties, Karagandinskaya 22 stood out for its high yields, i.e. 4.0 t/ha [30]. In terms of yield, the Sary-Arka (3.7 t/ha) and Stepnaya 60 (3.4 t/ha) varieties should also be noted, with the average value of their biological yield of (3.00 ± 0.75) t/ha. In modern conditions of agricultural production, an increase in grain yield should be combined with adaptability, ecological plasticity and stability of varieties in various environmental conditions [28].

Thus, in mid-ripening varieties of spring soft wheat, we established a close positive relationship between the length of the vegetation period and the grain yield, the number of nodal roots and the productivity of plants in years with favorable weather conditions. A correlation was also found between the grain yield and the quantitative characteristics — the number of kernels per ear and the thousand-kernel weight. The main elements of the crop structure in the studied varieties were the number of plants per 1 m^2 (230-282 pcs./m^2), the number of productive stems (201-333 pcs./m^2), the number of kernels per ear (20-30 pcs.), the thousand-kernel weight (36.4-49.6 g). In the studied collection, the Karagandinskaya 22, Sary-Arka and Stepnaya 60 varieties were considered optimal in terms of grain yield for the steppe zone of the Akmola Region.

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METHODS OF LABORATORY ASSESSMENT OF POTATO CULTIVARS FOR RESISTANCE TO BACTERIAL BLACKLEG AND TUBER SOFT ROT
(review)

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Abstract

Bacterial blackleg and soft rot of potato caused by *Pectobacterium carotovorum* subsp. *carotovorum*, *P. atrosepticum*, and *Dickeya* spp. are among the most harmful diseases of potato. Average annual yield losses of potato caused by these bacteria make 10-15 %, but during epiphytoties they may exceed 50 %. Existing commercial potato cultivars do not possess high resistance to these diseases, since the most of current breeding programs do not consider this trait as a priority one. However, in recent years, global potato losses caused by blackleg and soft rot significantly increased that provided a growing demand for resistant cultivars and also for efficient methods for laboratory assessment of breeding material and new cultivars and hybrids for their resistance to these diseases. Weak correlation between the resistance of potato plants to the blackleg and soft rot results in the need of a parallel assessment for each of these two traits (R. Czajkowski et al., 2011). The choice of a preferable assessment method depends on the purpose of a study and the availability of biological material and required equipment and facilities. In large breeding centers, the assessment of potato resistance to the blackleg may be performed in vitro using potato explants. This approach is characterized by good reproducibility and reliability and provides a possibility for rapid large-scale production of revealed resistant genotypes (I. Hudák et al., 2006). If tested plants are planned to be used in further studies, then the detached leaf assay should be chosen (A. Sima et al., 2015). Results of this assay are usually in agreement with the results of field resistance assessment. The assay is preferable for a large-scale screening of resistance donors among wild Solanum species or transgenic potato lines. Breeders, who work with true potato seeds and mini-, micro-, and usual tubers, can use the method for potato resistance assessment under controlled conditions (V.S. Bisht et al., 1993). Tuber resistance to soft bacterial rot can be assessed using the vacuum infiltration method (M. Koppel, 1993) or the method of tuber or slice inoculation under anaerobic (R.A. Bain et al., 1988) or aerobic (I. Hudák et al., 2009) conditions. For aerobic conditions, the assessment may be carried out using whole tubers or their slices; in the last case, the duration of the experiment is significantly reduced (K.S. Tseng et al., 1990). The assessment criteria include the size of tissue necroses in the point of inoculation, weight or volume of affected tissues, and the ratio between the weights of healthy and affected tissues or between the areas of affected and healthy tuber surface. The choice of an assay and an assessment criterion depends on the purpose of the study and available resources. Comparison of results obtained by different methods may be incorrect. Planning and implementation of experiments on the assessment of tuber resistance to soft rot requires a standardization of some factors influencing on the final results; non-observance of this condition will make a comparison of obtained results impossible. Such crucial factors include the species of the inoculum, temperature of tuber tissues and bacterial suspension during inoculation stage, temperature of incubation after inoculation, and the point of inoculation or the part of tuber from which a slice was obtained. In the case of assessment of potato resistance to blackleg, the study of such factors was not conducted. This paper reviews in details advantages and disadvantages of the described approaches and factors and conditions able to influence on the results of assessment and on the possibility to compare results obtained in different experiments.

Keywords: potato, bacterial diseases, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum*, *Dickeya* spp., resistance assessment, bacterial blackleg, soft rot

Modern cultivars have a high genetic yield potential, which is, however, only realized by 30-50% under production conditions [1, 2]. One of the main causes is thought to be diseases caused by phytopathogenic microorganisms. In this regard, the assessment of persistance of source materials and cultivars and hybrids thereof to infectious diseases is an important place in plant breeding. It objectively speaks for their breeding value and applicability in various regions of Russia. Information on assessment methods is necessary for researchers to select new cultivars or perform registration tests.

The purpose of this article is to review foreign publications on methods for assessing the persistance of potatoes to bacterial diseases caused by *Pectobacterium carotovorum* subsp. *carotovorum*, *P. atrosepticum* and *Dickeya* spp. Its relevance is due to the increased economic importance of these pathogens in recent years, and almost full absence of publications on this subject in the Russian language.

Potatoes are one of the most important crops in the Russian Federation, however, having quite low yield (15-20 t/ha) [3]. Potato diseases associated with phytopathogenic bacteria rank second after late blight in economic terms [4]. Unlike viruses, bacterial infection is able to persist even during in vitro production of pathogen-free plantlets via meristem culture and degrade the quality of seeds and reduce yield [5]. Bacterial lesions cause weakening and death of plantlets during growth, rotting of tubers in the soil and during storage. Annual yield loss from bacteriosis is 10-15% and it can reach up to 50% during high epiphytotic periods [6]. Over the past period, potato losses from bacteriosis have increased significantly in the world. This is due to climatic changes conducive to the reproduction of phytopathogenic bacterial flora and the emergence of new harmful bacteria, the widespread switching to mechanized harvesting of potatoes, which increases the risk of damage to tubers, and the lack of timely and accurate diagnosis of bacteriosis [7].

One of the most harmful bacterial diseases of potatoes is the so-called 'blackleg" accompanied by soft rot of tubers. Damage to the underground parts of stolons at the beginning of the vegetation period leads to slower growth and gradual top necrosis, whereas the development of the infection in the middle of the vegetation period appears in the form of blackening and necrosis of the root part of the stems, accompanied by the decay and yellowing of the leaves [8]. Generally, the affected stems die back until the tubers are fully ripe, which negatively affects crop quality. Tubers harvested from the affected plants are often infected with a causative agent of the disease, which leads to the development of soft rot symptoms during the vegetation or storage period. Tubers can be infected from the affected stolons or lentils, when bacteria from the affected parent tuber enter the root zone [8].

Blackleg pathogens include several distinct, however, closely related species of pectolytic bacteria, originally called *Erwinia carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*. However, based on the results of phylogenetic analysis using the nucleotide sequences of the 16S ribosomal RNA gene, it was proposed to isolate them and some other *Erwinia* species into a separate *Pectobacterium* genus and re-name them, respectively, to *Pectobacterium carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *atrosepticum* and *P. chrysanthemi* [9]. Subsequently, *P. carotovorum* subsp. *atrosepticum* atrosepticum was identified as a separate *P. atrosepticum* species [10], and *P. chrysanthemi* was transferred into a new genus called *Dikeya* [11].

P. carotovorum subsp. *carotovorum* and *P. atrosepticum* are widespread potato pathogens. *Dikeya* species were first reported as causative agents of potato stem rot in the Netherlands in the early 1970s [8]. At about the same time, a

Dickeya sp. was also identified as the causal agent of a blackleg disease of potato in Japan and tuber soft rot in Australia [12, 13]. Subsequently, the association of *Dickeya* spp. with above mentioned potato disease was reported from France, Israel, and South Africa [14-16]. Since 2004, *Dikeya* spp. has spread throughout Europe, which is associated with an increase in the frequency of hot spring/summer seasons in the northern regions, as well as the emergence of a new *D. solani* biovar [17-19]. In recent years, this phytopathogen has become a serious problem for the whole of Europe [20-23] and countries importing seed potatoes from Europe [24-26]. Currently, the *D. dianthicola* bacteria are included in the EPPO (European and Mediterranean Plant Protection Organization) A2 list of pests recommended for regulation as a quarantine pest with limited distribution in the EPPO member countries [17]. In Russia, *Dikeya* spp. strains were first reported in 2009 [27].

Few potato varieties as well as wild potato species are resistant to the disease and none are immune to pathogens of blackleg and soft rot of tubers [28, 29]. Currently, none of the commercial potato varieties is highly resistant, because this feature has not been prioritized in most existing breeding programs [30]. The resistance of potatoes to the manifestation of blackleg symptoms and the resistance of tubers of the same varieties or hybrids thereof to soft tuber rot do not always correlate with each other. This complicates the breeding for disease resistance, because weak correlation between the resistance of potato plants to the blackleg and soft rot results in the need of a parallel assessment for each of these two traits [31-34; J. Dobránszki, personal communication).

Generally, field tests for resistance are more labor-intensive and complex, therefore considerable attention is paid to the development of fast and easy-to-use laboratory methods. There are currently no standards for such methods, although most of them use the same inoculum concentration — 1×10^8-2×10^8 CFU/ml, which only differs significantly for a few variants. The established methods vary depending on the object, technique, assessment scale and can be divided into several groups.

The assessment of potato resistance to the blackleg may be performed in vitro using potato explants. When using this technique, the cultured explants are artificially contaminated. Plant age and methods of contamination may vary. The use of explants free of viral and fungal pathogens excludes variations in sensitivity to the blackleg pathogen associated with infection.

I. Hudák et al. [35, 36] used potato plants of different varieties grown on a hormone-free Murashige-Skoog (MS) medium under controlled conditions at a temperature of 20-22 °C and a 16-hour photoperiod. In 3 weeks, plant stalks were inoculated with a help of a tweezer moistened with *E. chrysanthemi* bacterial suspension or sterile distilled water (control). After 1 week after inoculation, the development of symptoms of the disease was assessed. The plants were divided into five groups depending on the severity of the disease [35]: 1 — no symptoms; 2 — 1-25% of decayed leaves; 3 — 26-50% of decayed leaves; 4 — 51-75% of decayed leaves; 5 — 76-100% of decayed leaves.

For each variety, the infection index F_i was calculated by formula:

$$F_i = \frac{\sum\left[(N_1 \cdot 1)+(N_2 \cdot 2)+(N_3 \cdot 3)+(N_4 \cdot 4)+(N_5 \cdot 5)\right]}{\sum N},$$

where N_1-N_5 is the number of affected plants in each of the 5 groups, $\sum N$ is the total number of plants used in the test. According to the proposed methodology, a variety was considered stable at $1 \leq F_i \leq 2$, moderately susceptible at $2 < F_i \leq 3$, and very susceptible at $F_i > 3$.

Two other options for using potato explants to assess a variety's resistance to *P. atrosepticum* are presented in I. Hudák's research [37]. For both cases, they took 30-day plantlets cultured in a MS medium under conditions similar to those described above. In the first case, the plant was inoculated with a bacterial suspension of *P. atrosepticum* using a sterile toothpick, which was first immersed into the suspension, and then pressed against the top of the plant. In the second case, the plants were cut at the level of the second node, then they were dipped into a bacterial suspension and finally placed on a MS medium (6%). The degree of infestation was measured after 72 hours on a 6-point scale: 1 point — 0% of decayed leaves (highly resistant), 2 points — 1-25% (resistant), 3 points — 26-50% (moderately resistant), 4 points — 51-75% (moderately susceptible), 5 points — 76-99% of decayed leaves (susceptible), 6 points — 100% (highly susceptible). The results of the two measurements were consistent with each other and with the results of tests performed in the greenhouse. Both resistant (up to 4 points) and susceptible (> 4 points) varieties were identified, among the tested plants.

Measurement of resistance to the blackleg using potato explants is characterized by good reproducibility and reliability and well suited for application in breeding centers, when there are a significant number of explants. Once we have found a resistant clone by using potato explants we are provided a possibility for rapid large-scale production of revealed resistant genotypes.

Assessing the potatoes resistance to blackleg in vitro using separated leaves. If tested plants are planned to be used in further studies, then the detached leaf assay should be chosen, which is also suitable for breeders who are not able to work with explants.

P.S. Bains et al. [33] worked with the plants cultured from explants, true seeds or microtubers on the soilless Ter-ra-Lite 2000 medium under controlled conditions for 5-6 weeks. Sterile silica sand was poured at the bottom of the vessels with lids and moistened with inoculum (10^5 CFU/ml). Five leaves with petiolules 85 mm long and 2-3 mm in diameter were cut from a plant of a tested variety and placed in sand down to a depth of 5 mm. Then, the vessels were left for 72 hours at 20 °C and a 16-hour photoperiod, with lids closed. Pathogen resistance was measured by the length of the infected area of the cutting using a 4-point scale: 1 point — 0-5 mm (highly resistant), 2 points — 6-10 mm (stable), 3 points — 11-50 mm (susceptible), 4 points — > 50 mm (highly susceptible). A similar, but slightly modified approach was applied to measure transgenic potato resistance to blackleg [38].

The advantages of this approach include high reproducibility, suitability for mass screening and technical simplicity. The approach is highly sensitive and provides satisfactory results at an inoculum concentration of 10^4-10^5 CFU/ml. Results of this assay are usually in agreement with the results of field resistance assessment [39]. It should be noted, however, that in the two studies mentioned, all potato varieties were assessed as susceptible and highly susceptible varieties. Resistant and highly resistant samples were found only among wild-growing *Solanum* species and somatic hybrids of potatoes with related species. The assay is preferable for a large-scale screening of resistance donors among wild *Solanum* species or transgenic potato lines.

Potato resistance assessment under controlled conditions. This method involves the inoculation of a bacterial suspension of plants grown in pots under controlled greenhouse conditions.

O.A. Hidalgo et al. [31] grew several clones of *S. tuberosum* subsp. *andigena* from tubers planted in pots with a mixture of peat, sand and soil (1:2:3)

for 4 weeks at 25-30 °C. Then the plantlets of a height of about 35 cm were inoculated by pricking a toothpick dipped in a suspension of bacterial cells of *E. chrysanthemi* for 5 minutes in the axil film of the 3rd leaf. The inoculated plants were placed into closed humidity chambers (28-30 °C, humidity ~ 100%), for 24 hours, and moved transferred to a greenhouse and kept for 3 days at 25-30°C under high humidity conditions. After that, the resistance of the plants was assessed by the length of the affected part of the stem. The degree of difference in the resistance was found to depend on the concentration of the bacterial suspension. When using an inoculum of 5.5×10^7 CFU/ml, the clones with the length of the affected stem area of 5-20 mm were rated as resistant clones, the clones with the length of the affected stem area of 33-36 mm were rated as moderately resistant clones, and the clones with the length of the affected stem area of 70-85 mm were rated as susceptible, and the clones with the length of the affected stem area of 118-128 mm were rated as highly susceptible.

A clearer rating scale was given by A. Sima [37]. Potato seedlings were rooted on a sand substrate for 14 days, then transplanted onto the soil and grown in the greenhouse for another 14 days, after which the stems were inoculated by pricking a sterile toothpick dipped in a suspension of *P. atrosepticum* at a height of 5 cm from the soil. The stem was wrapped with Parafilm laboratory tape to prevent it from drying out immediately after pricking. Infected plants were kept in a greenhouse for 21 days under high humidity conditions, after that, the length of the affected part of the stem was measured. The symptoms were assessed using the following scale: 1 point — 0-19 mm, 2 points — 20-49 mm, 3 points — 50-79 mm, 4 points — 80-109 mm, 5 points — 110 mm, 6 points — death. There is a high degree of compliance with the results obtained in the in vitro assessment of the same varieties.

A variation of the described approach is provided in the work dedicated to the production of transgenic potato plants resistant to bacterial and fungal diseases [40]. Transgenic plants derived from explants were grown on the soil for 3-4 months in climatic chambers (at 20 °C, 16-hour photoperiod), and then inoculated by injection with a bacterial suspension into the root bud. The degree of disease was assessed at regular intervals: 1 point — no symptoms, 2 points — minor manifestations of leaf chlorosis, 3 points — mature leaf chlorosis and necrosis, 4 points — stem necrosis and decay, 5 points — death of the plant (see the original article with photo).

The approach aimed at assessing the resistance of plants under controlled conditions can be convenient when using botanical potatoe seeds and mini-, micro-, and ordinary tubers as planting material. The approach should not be applied if there is a limited amount of seeds.

Approaches for assessing tuberous resistance to soft rot. *Vacuum infiltration.* The most technically complicated tuber inoculation approach. Surface sterilized tubers are immersed in a bacterial suspension and incubated for 1 min at a negative pressure of 80 kPa [41]. After that, the tubers are placed in plastic bags with a small amount of distilled water and incubated for 3 days at 25 °C, then weighed, washed out with a stream of water affected by rotten tissue, and weighed. The degree of damage is assessed by the ratio of the weight of the affected and healthy tissues. This approach helps bacteria penetrate into the tissues of tubers through lentils and aims to simulate the condition of tubers in moist soil moist tubers during storage [42]. In addition to technical complexity, the approach is limited by poor reproducibility [43].

Spot inoculation in an anaerobic environment. After the tubers have been washed and dried, they are provided with holes drilled 10 mm deep along the

longitudinal axis. A standardized concentration suspension of bacterial cells is applied to the bottom of a hole, and the hole is then sealed with a mixture of melted wax and vaseline. Tubers are then placed in plastic boxes, filled with nitrogen, sealed and kept for 6 days at 15°C, after that, the width and depth of tissue damage is measured. The criterion for assessing the resistance of the tuber is the degree of tissue damage (P), calculated by formula: $P = [W/2 + (D - 10)]/2$, where W is the width, D is the depth of tissue damage. It is also possible to assess the damage by the ratio of the weight of the affected and healthy tissue.

To apply a simplified version of this technique one should inoculate the sterilized tubers by 2-3 injections with a microsyringe to a depth of 2 cm [31]. All injection sites are placed on one side of the tuber and covered with a layer of vaseline, after that the tubers are wrapped in wet filter paper and plastic film, placed in plastic boxes and kept for 72 hours at 25 °C. After that, the tubers are cut through the points of inoculation and the width of the affected area is measured. The authors propose the following scale to assess the resistance: 0 points — width of tissue necrosis at the site of inoculation of a tuber 0-5 mm (resistant), 1 point — 5.1-10 mm (moderately resistant), 2 points — 10.1-15 mm (susceptible), 3 points — > 15 mm (highly susceptible).

Another option is to incubate solid tubers previously immersed in a bacterial suspension in chambers with a constantly maintained introductory suspension for 20 minutes, which ensures the continuous presence of a water film on the tubers [45]. As a result, the oxygen content in the tuber tissues decreases rapidly, which grows into a stronger manifestation of infection. The damage area is assessed (as a percentage of the total surface tuber area) after 96 hours of incubation at 20 °C.

This approach involving the incubation of tubers under anaerobic conditions ensures the progressive development of the infection and excludes the formation of wound periderm in potato. Therefore, the resistance of varieties is lower than that when assessed by using other approaches [46]. This approach helps assess how well the tuber tissue can serve as additional food basis for the pathogen, simulate tuber storage and transportation conditions, and select a highly resistant breeding material.

Inoculation of tuber slices and cut-outs in an aerobic environment. The inoculation of tuber slices in an aerobic environment is used to simulate the tuber response to a wound infection under aerobic conditions that are similar to the tuber post-harvest storage conditions, and to assess the tuber wound repair ability [44]. After the tuber surface has been sterilized, three slices of appr. 10 mm thick are cut out from the tuber and placed at right angles to the tuber longitudinal axis. Cavities with a depth and diameter of 5 mm are made in each slice, then left to initiate the formation of wound periderm for 6 hours, and then inoculated with a bacterial suspension. Pallets with slices are wrapped in wet filter paper and kept at 15 °C for 3 days. Tuber resistance is measured by the diameter of the affected tissue.

In later studies, the authors inoculated the slices immediately after cutting, without waiting for the formation of wound periderm [46-48]. There is also an option, where filter paper disks with a diameter ~ of 1 cm soaked in a bacterial suspension are placed on slices or halves of tubers [46, 47, 49]. Here is another approach, which is described by I. Hudák et al. [48]. After the tuber surfaces have been sterilized, cylindrical cut-outs were obtained from the tubers and cut into disks with a height of 1 cm (20-25 disks per option). The weighted discs were put into sterile Petri dishes on wet filter paper, covered with a 0.1 ml bacterial suspension or water and incubated at 26 °C for 24-26 hours. The affected tissues were then washed from the discs, and the disks were dried out and

weighed, and the resistance was assessed using the ratio of the weight of the remaining healthy tissues and the initial weight as a criterion: 1 point — < 5% (highly susceptible), 2 points — 5.1-10% (susceptible), 3 points — 10.1-15% (moderately susceptible), 4 points — 15.1-20% (poorly susceptible), 5 points — 20.1-30% (moderately resistant), 6 points — > 30.1% (resistant).

By inoculating slices or cut-outs we reduce the amount of testing time as compared to the inoculation of solid tubers. This approach shows high variability of results and lack of standardization for experimental conditions [46].

Inoculation of solid tubers in an aerobic environment. In recent years, a variety of techniques to inaculate and incubate solid tubers under aerobic conditions have developed. Tubers can be inoculated by pressing a plastic drop-tube tip containing a small amount of inoculum into a tuber to a depth of 1-1.5 cm [50-51]. Tubers are placed in high-humidity plastic containers with wet filter paper or wet chambers for 3 days [51] or 6 days [50] at 20-22°C and then assessed using the following scale: 0 points — no tissue necrosis at the inoculation area (resistant), 1 point — necrosis width < 2 mm (susceptible), 2 points — 2-10 mm (susceptible), 3 points — > 10 mm (susceptible).

T. Thangavel et al. [52] propose two more inoculation options. Firstly, the tubers were intentionally damaged by a falling weight with a steel tip, and then immersed for a short time into the pathogen suspension. Secondly, a hole with a depth of about 10 mm was made by a corkscrew in the tuber, at the bottom of which a bacterial suspension was then applied. In both cases, the tubers were weighed in 5 minutes after inoculation, wrapped in plastic film, distributed to plastic bags with a damp filter paper inside and tightly closed. After a 14-day, incubation in the dark at a temperature of 20-25 °C, the decayed tissues were washed out with water, the tubers were dried out and weighed, and the ratio of the weight of the decayed tissues and the healthy weight was measured. The damaged tissues can be used to assess tuber resistance. The approach is to determine the amount of damage by measuring the volume of water that is needed to fill the cavity remaining after careful removal of the decayed tissue [40, 53].

Factors influencing the assessment of blackleg and soft tuber resistance. When performing laboratory assessment of the resistance of potato plants to said diseases, it is necessary to take into account that, although the resistance is mainly determined by genetic characteristics [28], the degree of its manifestation depends on a number of external factors. These include seed quality, tuber physiology, the amount and virulence of inoculum, tuber infestation by other pathogenic microorganisms, weather conditions (temperature and humidity) during growing and harvesting, damage to tubers during harvesting, transportation and storage conditions. Therefore, a very strict observance of one and the same experimental conditions is a must. See below the main factors that can influence the outcomes of laboratory testing and are subject to standardization at the planning and testing stages.

Firstly, the degree of resistance depends on the inoculum origin. There were shown differences in the potato clone resistance to the blackleg caused by artificial inoculation with three different pathogens, *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum* and *E. chrysanthemi* [31], and differences in the tuber response to the infestation by *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* [54]. Secondly, the spread of bacterial infection on tubers depends on the tissue temperature, and the temperature of the suspension, if inoculated by immersion in bacterial suspension. For example, the degree of damage caused by *P. carotovorum* subsp. *carotovorum*, was higher for tubers with the temperature of 23-24 °C at the time of inoculation than for cold tubers (4 °C) or excessively warm

tubers (26 °C) [55]. In the same study, it was shown that the maximum spread of tuber rot occurs when warm tubers are immersed in cold (10 °C) suspension. Thirdly, the extent of the damage can significantly depend on the incubation temperature after inoculation. For example, the increase from 20 to 30 °C resulted in the increase of the diameter of the affected potato slice area when inoculated with *P. carotovorum* subsp. *carotovorum* and *E. chrysanthemi* by about 25%. It should be noted, however, that in the case of *P. atrosepticum* the change was only 2.5% [47]. Finally, when performing tuber-slice tests, the degree of tissue damage strongly depends on the area from which the slice was cut out (or the inoculation point on the slice). Maximum tissue damage was observed for the slices obtained from the middle of a tuber, whereas the slices cut out from the apical parts turned out to be more resistant [46]. This phenomenon wad proved at different varieties of tubers. Further research showed that the diameter of the damage was significantly higher in the inoculation of the core part of the slice, but not the peripheral (cortex) one. For example, in one of the tests, the diameter of the damage for the two inoculations was 23.2 and 3.1 mm, respectively.

Therefore, there is little correlation between potatoes resistance to blackleg and soft tuber rot. For this reason, individual tests are required. Choosing the right assessment approach depends on the goals of the researcher and their access to the biological material, equipment and premises. Assessment of the potato resistance to the blackleg can be performed in vitro on explants or separated leaves, and on whole plants under controlled conditions. When assessing tuber resistance to soft bacterial rot, one can apply various approaches simulating these conditions or any other environment favorable to the spread of infection, by using either whole tubers or slices or cut-outs. These approaches include vacuum infiltration, spot inoculation in an anaerobic and aerobic environment. The size of tissue necrosis at the inoculation site, the weight or volume of affected tissues, and the ratio of the weight of healthy and affected tissues or the area of the affected surface and the surface of the whole tuber can serve as a criterion for assessing tuber resistance. It must be noted that the described approaches to the analysis of resistance to soft rot do not always give similar and comparable results, since they presumably activate different plant defenses. Hence, a comparison of the results obtained by applying various approaches to assessing tuber resistance may turn out to be irrelevant. When planning and practicing the assessment of tubers for resistance to soft rot one should take into account that the lack of standardized benchmarks for the factors that may influence outcomes will hinder any objective comparison among the outcomes. It should yet be noted, that the problem requires further study, because said factors have practically not been studied in the context of the plant resistance to the manifestation of blackleg symptoms.

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POTATO PHYTOPATOGENS OF GENUS *Dickeya* — A MINI REVIEW OF SYSTEMATICS AND ETIOLOGY OF DISEASES

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Abstract

In recent years, plant growers in Russian Federation have met significant changes in species of bacterial pathogens causing economically harmful diseases of potatoes that is associated with the import of infected planting material, recent climatic changes favorable for bacterial disease development, over-wintering of the pathogens and their vectors (insects, mites and nematodes), and with lack of bactericidal pesticides for integrated plant protection. Damage of potato plants (*Solanum tuberosum* L.) by *Enterobacteriaceae* family is one of the greatest problems in production of seed and food potatoes. The bacteria cause a black leg, wet rotting of the stem in the field, and soft rot of potato tubers in storage. In temperate climate, the bacterial diseases of potatoes was usually caused by two species of genus *Pectobacterium*, *Pectobacterium atrosepticum* as a pathogen of black leg of potato, and *Pectobacterium carotovorum* causing a soft rot of potato and different vegetable crops (A.N. Ignatov et al., 2015). However, recently, many countries have faced the spreading on potato fields of new enterobacteria of genus *Dickeya*, which has been normally considered as pathogen of ornamentals and vegetables, particularly in countries of tropical and subtropical climate. A detailed study of genus *Dickeya* has shown that this diverse group of bacteria affects a number of plant species, including many economically important crops (I.K. Toth et al., 2011). The strains differed in attacked host plants, and phenotypic properties. It was found that strains isolated from European potato fields in years 1979-1994 were mainly related to *D. dianthicola*, the species well-adapted to temperate climatic regions. However, since 2005, the variants of *Dickeya*'s biotype III, referred to the new species *D. solani* were detected on potato in Europe, and soon became one of the most aggressive pathogens of this crop. Clarification of the taxonomic position and diversity within species of the genus *Dickeya* (*D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca*, *D. zeae*, *D. solani*) gives a chance for development of new methods of diagnostics and control measures against these pathogens (L. Tsror et al., 2011). Except for *D. dieffenbachiae*, all the species of this genus can affect potatoes. Infection of *D. dianthicola* and *D. solani* has been already reported in some regions of the European part of the Russian Federation (A.N. Karlov et al., 2010, 2011; A.M. Lazarev, 2013), and genome sequencing of the isolated bacteria showed their identity with strains of this genus isolated in Western Europe and Latin America (S.V. Vinogradova et al., 2014). The spreading of these pathogens abroad and in Russia, data on taxonomic position and description of their biological properties, and sources of infection, created ground for development of control measures against them. It is believed that *D. dianthicola* and *D. solani* have aroused as potato pathogens moving from vegetable crops in the early 1990s. Now they are striking plants in European countries, USA, South America, Africa and Asia. *D. dianthicola* and *D. solani*, first described at the territory of the Russian Federation in 2009, cause serious potato losses in Russia in recent years. In 2009-2013, the annual two-fold increase of contamination of seed potatoes by these pathogens was documented. Thus, in just 4

years, the prevalence of pathogens of the genus *Dickeya* in potato seed lots in Russia increased from 3 % to 26-28 % (A.N. Ignatov et al., 2015). Control of these pathogens on potato is based on the rejection of contaminated material and prevention of contamination at all stages of the technological cycle of seed potatoes. Potato varieties resistant to these pathogens have not been yet discovered.

Keywords: potatoes, bacterial diseases, blackleg, soft rot, *Pectobacterium*, *Dickeya*

In recent years, in the Russian Federation significant changes in species of bacterial pathogens and their enhanced harmfulness have been noted. Primarily, it is associated with the import of infected seeds and planting materials, secondarily with climatic changes favorable for bacterial disease development, overwintering of the pathogens and their vectors (insects, mites and nematodes), and thirdly with lack of the chemicals with high bactericidal effect [1].

Lack of information about pathogens diversity makes it impossible to assess potential losses from disease and to choose the correct strategy for selection of protective measures.

We analyzed available information about spreading, harmfulness and genetic diversity of new potato pathogens from genus *Dickeya* causing bacteriosis which are of interest in developing diagnostics methods and phytopathogen control.

Black leg and wet rotting pathogens belong to the group of pectolytic enterobacteria including species of the genus *Pectobacterium* (earlier *Erwinia*). The complex of *P. carotovorum* species includes subspecies *P. carotovorum* subsp. *actinidiae*, *P. carotovorum* subsp. *brasiliense*, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum*. *P. atrosepticum*, *P. betavasculorum* and *P. wasabiae* are grouped apart from *P. carotovorum*. Several new species (*P. aroidearum*, *P. cacticida*, *P. parmentieri*) were described for certain host plants [2, 3]. Polymorphous species of phytopathogenic bacteria *P. chrysanthemi* [4] which previously was a member of the genus *Pectobacterium*, in 2005 was defined as a separate genus *Dickeya* [5] based on the complex of phenotypic traits and genetic analysis data.

In Russia, the prevalence of *Dickeya dianthicola* and *D. solani* causing significant yield losses of potato was first described in 2009 [6]. To date, pathogens of the genus *Dickeya* have been found in all Russian regions [1, 6-8]. In 2009-2013, the infestation increased 2-fold each subsequent year. In just 4 years, the prevalence of the genus *Dickeya* in seed potatoes increased from 3% to 26-28% [1].

Whole genome sequencing of two strains *D. solani* D12 and Dfil, isolated in Russia in 2009 [9], showed that they have almost complete homology with strain IP2222 (T) detected in the Netherlands [10]. So, the most probable cause of *D. solani* penetration to the Russian Federation is import of the seed potato from countries of Western Europe, where in 2007 an extensive black potato leg epiphytoty on seed fields was noted [11]. Russian strains of *D. dianthicola* also had high genetic homogeneity and proximity to typical culture isolated earlier in Latin America and Europe [3].

Original name of *P.* (*Erwinia*) *chrysanthemi* is due to the fact that the pathogen was first described as a causative agent of the bacteriosis in chrysanthemums [4]. Further it has been shown that these microorganisms cause plant diseases in plant of at least 16 dicotyledonous and 10 monocotyledonous families [5, 12, 13]. R.A. Lelliott and R.S. Dickey [13] divided *P. chrysanthemi* species into 6 pathovars based on their host specificity: *chrysanthemi, dianthicola, dieffenbachiae, paradisiaca, parthenii* and *zeae*.

DNA hybridization and biochemical characterization of pectinolytic bacteria had led to the separation of *P. chrysanthemi* species from the genus *Pectobacterium* into a new genus named *Dickeya* in honor of the outstanding microbiologist R.S. Dickey [5] who had devoted many years to this bacterium research [13,

14]. To date, according to the official microbiological LPSN (List of prokaryotic names with standing in nomenclature) [15], the genus *Dickeya* is clearly differentiate in the following species: *D. aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dadantii* subsp. *dadantii*, *D. dadantii* subsp. *dieffenbachiae*, *D. dianthicola*, *D. fangzhongdai*, *D. paradisiaca*, *D. solani*, *D. zeae*. All *Dickeya* spp. members, except for *D. paradisiaca*, are isolates from the cultivated and ornamental plants, including those imported to Russia from different countries [16].

The table shows host plants the most frequently affected by *Dickeya* spp. species, with synonyms.

Bacteria host plants of the genus *Dickeya* [11, 12, 14]

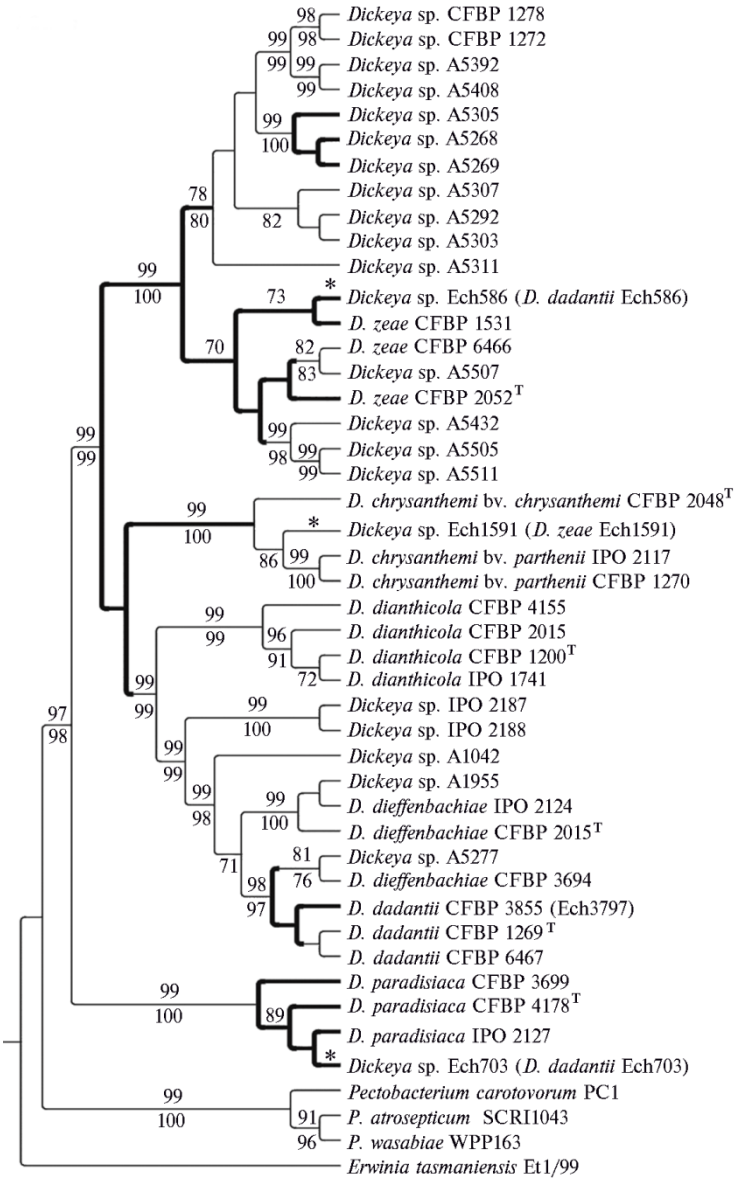
Species	Host plants
Dickeya dianthicola [syn.: *Pectobacterium* (*Erwinia*) *chrysanthemi*, biobars I, VII, IX; *E. chrysanthemi* pv. *dianthicola*]	Carnation Chinese, potatoes, tomato, chicory, artichoke, dahlia, hyacinth, iris, kalanchoe
D. dadantii [syn.: *P.* (*E.*) *chrysanthemi*, biobars III and VIII]	Pelargonium, potato, Chinese yam, pineapple, banana, carnation species, euphorbia, senpolia, maize, philodendron, scindapus (divel's ivy), ragwort, eryngium (sea holly), arrowhead vine
D. zeae [syn.: *P.* (*E.*) *chrysanthemi*, biobars III and VIII]	Maize, potato, pineapple, banana, tobacco, rice, Para grass, chrysanthemum, wheat, carnation species, cantante, ahmeya (ehmeya), scindapus, cabbage, diffenbachia
D. chrysanthemi bv. *chrysanthemi* [syn .: *P.* (*E.*) *chrysanthemi*, biobar V; *P.* (*E.*) *chrysanthemi* pv. *chrysanthemi*]	Chrysanthemum, chicory, tomato, sunflower, potato, carrot, patrenium (wild quinine), euphorbia
D. paradisiaca [syn.: *P.* (*E.*) *chrysanthemi*, IV биовар; *P.* (*E.*) *chrysanthemi* pv. *paradisiacal*; *E. aradisiaca*; *Brenneria paradisiaca*]	Banana
D. dieffenbachiae [syn.: *E. chrysanthemi*, biobar II; *P.* (*E.*) *chrysanthemi* pv. *dieffenbachiae*]	Dieffenbachia, tomato
D. fangzhongdai	Pear
D. aquatic	The host plant was not identified

Evolutionarily, genus *Dickeya* is monophyletic and represents a sister clade for the genus *Pectobacterium* (Fig.), and they differ substantially from other phytopathogenic and non-symbiotic enterobacteria [17]. In addition to species similar to the previously described *P. chrysanthemi* pathovars (*D. chrysanthemi*, *dadantii*, *dieffenbachiae*, *dianthicola*, *paradisiaca*, *zeae*), several European researchers defined strains of so-called biovar III, causing a new potato disease in Western Europe, as a new species *D. solani* [18]. *D. solani* is highly aggressive and causes typical symptoms of the potato disease (watery stem rot). Lately, the species D. aquatic which is often isolated from river water but still not detected in plants were identified [19], and the last of known species of the genus *Dickeya*, *D. fangzhongdai* causing pear disease, has been described recently [20].

D. dianthicola (called *E. chrysanthemi*) was first reported in the Netherlands in the 1972 [11]. Then the pathogen was detected in Israel [21], Sweden [2], Switzerland [22], Spain [23], Finland [24], France and UK [25], Poland [26], Greece [27] and Japan [28]. There were reports about *Dickeya* spp. presence in Scotland, Denmark, Hungary, Germany, and Belgium [5]. Withal, in most European countries, the losses associated with *D. dianthicola* remained low, except for Switzerland where the potato damage from *Dickeya* spp. was predominant as early as in 1992 [22]. During field tests in Finland [29] in comparing direct losses from *D. dianthicola* and *D. solani,* significant differences of tubers damage (5-6%) were not identified, but significant damage of stems with the second pathogen (73% vs. 20%) was noted. By the report of R. Czajkowski et al. [30], during a three-year study the differences in frequency of potato disease caused by *D. solani* and *D. dianthicola* was not detected. However, in another paper it was noted, that the losses from *D. solani* exceeded the harm from *D. dianthicola*, *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* [5].

Signs of new potato bacteriosis are similar to the black leg of stem and

the tuber soft rot caused by *Pectobacterium*. Unlike them, *D. dianthicola* causes tubers soft rot at higher temperature (at 27 °C), and *D. solani* causes mainly the stems wilting (watery rot) and the destruction of the vascular ring in the tubers, which remind the development of ring (*Clavibacter michiganensis* subsp. *sepedonicus*) or brown (*Ralstonia solanacearum*) potato rot. Strains *Dickeya* spp. are able to cause plants damage with lower pathogen load than *Pectobacterium*, they have more possibilities to spread through the vascular tissue and are more aggressive.



Phylogenetic tree of bacteria genus *Dickeya* constructed using *dnaA*, *dnaJ*, *dnaX* and *recN* gene sequences [17]. Strains *Dickeya* marked with an asterisk were initially defined as *D. dadantii* (Ech586, Ech703) or *D. zeae* (Ech1591). T means typical species strains. Tree branches differed in construction by some genes and combined sequences are indicated in bold. Bootstrap values ≥ 70 % are shown above branches for tree constructed by maximum parsimony method, and below branches for maximum credibility method.

Pre-germination development of tuber infection or its early post- germination appearance usually is followed by the damage of the mother (seed) tuber and causes plant losses. At the optimum pathogen temperature, the plants are

stunted, their leaves show yellowing, become small, hard and fold along the middle vein. The springs are located at an acute angle to the stem and growth up, and its lower part shows up from yellow-brown to dark in a color. Infected plants are pulled from the soil easily. At low temperature, infected plants may keep pace in growth with healthy ones, but with warming, their stems get dark suddenly from the ground to the upper leaves, leaves droop and gradually fade without changing color. Bacteria enter the tubers through the stolons which partly rot. Like in bacterial wilting and ring rot, genus *Dickey* pathogens stimulate growth of saprophytic and low pathogenic microorganisms on affected tubers. Usually infected tubers remain without symptoms until the spring, with hidden (latent) infection of stolons and the vascular ring until spring planting [2]. The quantity of young tubers with a latent infection depends on blackleg development on the plants during the growing season. In particular years up to 75% of the tubers of infected plants are carriers of phytopathogenic bacteria. Under unfavorable climatic conditions (dry cool weather), the disease does not develop, and bacteria from the planting tuber through the plant and stolons penetrate into the daughter tubers without visible signs [30].

J.K. Toth et al. [11] note the ability of *Dickeya* spp. species, unlike the cold-loving *P. atrosepticum*, to attack potato in subtropical regions (e.g., in North America, North Africa, Israel and southern Europe). It is suggested that global climatic changes (especially temperature rise in spring and autumn) can aggravate the problem of new species spreading, especially *D. solani*, which has caused the largest yield losses in potato during the last 5-6 years in Europe.

The blackleg and soft rot differ from others potato diseases visually. But in the hot and dry conditions bacteriosis symptoms caused by *D. dianthicola* or *D. solani* are easily confused with the disease caused by *Verticillium dahliae* or with accelerated plant ageing [21].

In plants attacked by *Dickeya* spp. pathogens, it is important to know the sources of infection to successfully restrict their further spread. There are two ways of spreading *D. dianthicola* and *D. solani* bacteria: i) transfer through the potato seed material and other host plants, and ii) transfer through rainwater, irrigation water and pest vectors. Due to a wide range of host plants (including ornamentals), *D. dianthicola* and *D. solani* can be further spread around the world not only in the seed trade and via food potatoes, but also through sale of flower crops [11]. Thus, there are reports of the *D. dianthicola* isolation from ornamental host plants in the USA, Colombia, Japan and New Zealand [11]. We are aware of the facts of *Dickeya* spp. survival in the bitter nightshade (*Solanum dulcamara*) in Sweden [31]. In Israel, asymptomatic plant samples of the local weed species were analyzed in details for the presence of *Dickeya* spp. and aise-weed (*Cyperus rotundus*) plants infected at a frequency of 6.7 up to 14.3% was detected [32].

There are reports of potato diseases caused by different *Dickeya* ssp. species: *D. chrysanthemi* is isolated in the USA and Taiwan [25, 26], *D. dianthicola* in Brazil [26], Peru [25, 28] and Zimbabwe [34], *D. zeae* in Australia and Papua New Guinea [25, 35]. Members of the *D. zeae* species were isolated from river water in Scotland and England, but were not found on potatoes [11]. *Dickeya* sp. bacteria isolated from potato plants in the Krasnodar and Stavropol territories belonges to *D. dadantii* (A.N. Ignatov, unpublished data).

Numerous researches indicate that at least one species of the genus *Dickeya* (*D. dadantii*) is closely associated with phytophagous insects. This species can colonize pea aphid (*Acyrthosiphon pisum*) and is pathogenic for three other insect species (*Drosophila melanogaster, Sitophilus ozuzae* and *Spodoptera littoralis*) [36, 37]. Infection transmitted by insects is possible even in a short-term nutrition on plants.

Compared to *P. carotovorum*, *Dickeya* spp. is less sensitive to cold, but better survives in water [35].

At present, the reliable information on the resistance of potato varieties to *Dickeya* spp. are absent. I.K. Toth et al. [11] showed that all tested in the UK varieties were susceptible to *D. dianthicola*. It was identified that *D. solani* can colonize potato roots within a day, regardless of the lesion presence. The pathogen was detected in stolon and stems 15 days after the soil contamination. It was also reported that *Dickeya* spp. colonizes the vascular system of potato plants more actively than *P. atrosepticum* [11, 38].

It was suggested to compare aggressiveness of tested *D. solani* strains by measuring the weight of the infected tissue using tuberous bioprobe (with 48 hour incubation at 30 °C) [39]. Nevertheless, the questions still remain about the *Dickeya* spp. interaction with other phytopathogens, especially in the latent infection, which is extremely important for the development of plant protection measures and parameters of seed potato infection while introducing new standards for growing.

With the widespread of *D. dianthicola* and *D. solani* on potato in most areas of our country, it is impossible to exclude in the near future the transition of these pathogens to tomato and other plants in greenhouses [11, 12, 14]. The biological characterization of bacteria from this genus is rather complete and a collection of their Russian strains is created. However, the species composition of genus *Dickeya* on the territory of the Russian Federation has not been determined in detail. Russian quarantine services should pay special attention to these microorganisms in view of imported potatoes from states with significant spreading of species *Dickeya* sp. Unfortunately, neither previously used, nor recently adopted regulations for assessment of seed potato infection with black leg pathogens (RF State Standard GOST 33996-2016) have made no distinction between *Dickeya* sp. and *Pectobacterium* spp., which can lead to rapid spread of *Dickeya* sp. in Russia.

Thus, we have summarized the available information on the spreading, damage and genetic diversity of new potato bacteriosis pathogens from the genus *Dickeya*. This information is of interest for the development of diagnostic tests to control seed purity and potato diseases. At the high infection load of seed potato with bacterial pathogens, it is extremely important to increase attention to new harmful species that have a significant potential for spreading and adapting to local conditions, but are not subjected to control by international and national quarantine organizations.

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ALLELE DIVERSITY FOR ACID VACUOLAR INVERTASE GENE *Pain-1* FRAGMENT IN POTATO (*Solanum tuberosum* L.) VARIETIES AND LINES

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Abstract

The economic efficiency of potato production depends not only on the yield quantity and quality, but also on the tuber storage conditions. The tuber nutritional and technical value is determined by the starch content. Under the cold stress, the starch degrades into reducing sugars (cold-sweetening), which significantly reduces the tuber quality. Plant invertases catalyze irreversible hydrolysis of sucrose into glucose and fructose. Nowadays it is definitely known that vacuolar acid invertase (*Pain-1*) plays a major role in the cold induced sweetening of potato tubers. In the present work genetic diversity and allelic polymorphism of *Pain-1* gene associated with important agronomical traits of potato tubers is characterized. Gene fragment (exon 5—exon 7) polymorphism was analyzed in 69 cultivars and lines of Russian and foreign breeding origin. In the *Pain-1* nucleotide sequences, 66 SNPs were identified, of which 25 SNPs (SNP_{1628}, SNP_{1648}, SNP_{1700}, SNP_{1709}, SNP_{1717}, SNP_{1724}, SNP_{1726}, SNP_{1738}, SNP_{1788}, SNP_{1794}, SNP_{1797}, SNP_{1808}, SNP_{1815}, SNP_{1818}, SNP_{1831}, SNP_{1837}, SNP_{1847}, SNP_{1861}, SNP_{1865}, SNP_{1872}, SNP_{1885}, SNP_{1886}, SNP_{1890}, SNP_{1907}, SNP_{1909}) were described for the first time. The studied fragment contains a significant replacement for SNP1544 (C/A) which correlates with an increased starch content in the tubers and is homozygous in the Kazakhstani varieties Ulan and Astana. In the exons, 27 out of 42 SNPs led to amino acid substitutions. Most accessions had single amino acid substitutions. The maximum substitution number (seven to eight) characterized the Zhukovskii ranii variety and the lines 165 and 162. No substitutions were observed in the Frittella variety and the line 84. Therefore, the common level of gene fragment polymorphism in the analyzed potato accessions was shown to be rather high. Among the analyzed sequences, 78 allelic variants were described, including 64 specific variants and 14 variants common for several accessions. The obtained data may be helpful in potato breeding for an increase in starch content.

Keywords: acid vacuolar invertase, *Pain-1*, exons, polymorphism, SNPs, amino acid substitution, potato breeding, starch content in tubers, cold-induced sweetening

Alongside with wheat, maize and rice, potato (*Solanum tuberosum* L.) is one of the most widely spread and most economically significant global food, forage and industrial crops. The potato production efficiency depends on not only the amount and quality of the harvest, but also on the subsequent conditions of its storage, which is usually effected at low temperatures so that to prevent loss of moisture, molding, sprouting and disease transfer.

The nutritional and technological value of potato tubers is determined by content of starch [1]. The plants' starch performs a storage function, because it does not produce any increased osmotic pressure, being chemically inert (as distinct from glucose and sucrose) [2, 3]. However, it is starch that is "a weak link"

in storage of tubers, because under the influence of low temperatures, significant changes occur in carbohydrate metabolism [4], namely, sucrolytic enzymes. It results in decomposition of starch into simple sugars (glucose and fructose), which take part in maintenance of intracellular osmotic pressure, thus increasing resistivity of plants to low temperatures. At temperature below +3 °C the protective reactions of tubers to overcooling activate, and starch starts decomposing intensively, which is accompanies by accumulation of reducing sugars (cold-induced sweetening) [5, 6]. As a result, due to worsening of palatability traits of potato tubers their commercial qualities deteriorate as well [7, 8]. In addition, during heat treatment (cooking boiled potatoes or potato chips) the reducing sugars interact with free amino acids, causing change of colour of the product and accumulation therein of a carcinogen, the acrylamide [9-11].

The search of nucleotide and amino acid replacements associated with accumulation of starch and sugars in tubers and absence of cold-induced sweetening is very significant for selection.

A small family of vegetable invertases, which include cell wall, vacuolar and cytoplasmic invertases, catalyzes not-reversible sucrose splitting to glucose and fructose. It has been clearly shown that acidic vacuolar invertase coded by gene *Pain-1* plays a leading part in cold-induced sweetening of potato tubers. Inhibition of gene *Pain-1* decreases accumulation of reducing sugars at low temperatures [12-16]. Potato (*Solanum tuberosum*) gene of acidic vacuolar invertase is identified, its structure and expression are studied, and single nucleotide substitutions (SNPs) are found, which can exert the decisive influence on the enzyme activity [17, 18].

Previously it has been shown that the maximum number of polymorphic sites is detected in C-terminal region (exon 5—stop codon), which has been studied well enough for foreign varieties; moreover, in the said region SNPs associated with the amount of starch in tubers have been discovered [17, 19]. Analysis of polymorphism of this element for a set of 19 potato varieties identified nine allelic variants [20].

The search of new allelic variants of gene *Pain-1* determining commercially significant attributes of potato and the assessment of the occurrence frequency of such variants is deemed important for optimization of the selection process [21-23].

In the presented research, based on the analysis of nucleotide and amino acid polymorphism, 78 allelic variants were identified for fragments of gene of acidic vacuolar invertase *Pain-1* for varieties and lines of potatoes, which have not been characterized for this attribute previously, and the presence of 42 SNPs is demonstrated; 34 of them have been described for the first time.

The research objective was analysis of C-terminal region of acidic vacuolar invertase *Pain-1* for 69 varieties and lines of potatoes of different origin, which are now used in selection programs.

Techniques. The allelic polymorphism of *Pain-1* acidic vacuolar invertase gene fragment was studied for 69 potato varieties, including 55 varieties of domestic and foreign selection, as well as 14 lines used now in selection programs (Lorch Potato Research Institute). The tubers were sprouted in a greenhouse in light and temperature regime 8/16 hours and 16/22 °C (night/day) with illumination intensity 10-12 klx. The selected specimens were characterized by different starch content and unequal resistance to abiotic factors.

The nuclear DNA was extracted by the protocol suggested by K. Edwards et al. (1991) from fresh leaves according to description [24]. For amplification of *Pain-1* gene fragment a pair of primers IV5exF (GAAGCCTCATTTGAAGTGGAC)—IVendR (AATGTATGGGTTCCTGGAAACCG) was used.

PCR was performed in 15 μl reaction mixture. It contained 1× buffer solution with 50 mM Trisc-HCl (pH 8.6), 50 mM KCl, 0.1% Tween 20 (Dialet Ltd, Russia), 1.5 M $MgCl_2$, 20 mM dNTP, 10 μM of the respective primer, 0.25 U Taq DNA-polymerase and ~ 100 ng of genomic DNA. The amplification was carried out with a Mastercycler gradient device (Eppendorf, Germany) using reagents kit produced by Dialat Ltd. (Russia). Temperature/time profile of PCR: 5 min at 94 °C; 35 cycles: 30 s at 94 °C, 40 s at 57.5 °C and 1 min at 72 °C. Final elongation: 1 min at 72°C. The PCR products were analyzed by electrophoresis in 1% agarose gel (LE 2 Agarose, Helicon, Russia) in 1× TBE-buffer solution with ethydium bromide at the field intensity increasing from 70 to 100 V/cm. The results were documented in BioDoc II system (Biometra GmbH, Germany). Commercial 1 Kb and 100 bp DNA markers were used as fragment size standard (Thermo Fisher Scientific, USA). The amplified fragments were sequenced with the same primers (Applied Biosystems 3730 DNA Analyzer, Applied Biosystems, USA).

Balancing, translating and analysis of polymorphism of nucleotide and amino acid sequences were performed with software MEGA 7.0 (25). The sequence from NCBI database (GeneID:102577489) was used as a reference.

Results. Nucleotide polymorphism of *Pain-1* gene fragment. A fragment of C-terminal region containing the sequence from exons 5 to 7 was amplified and sequenced in the studied specimens. The length of the obtained sequences of *Pain-1* in all analyzed specimens was the same and amounted 707 bps. Exon 7 was the longest one (199 bps), and exon 6 was the shortest one (91 bps). No indels have been found in the sequence of analyzed specimens, while in previous studies for Bryanskii rannii variety we have demonstrated the presence of 11-nucleotide deletion (CAAGCTTATAT) and mononucleotide insertion (T) in intron 6 [20].

The polymorphism level of the analyzed sequences of acidic vacuolar invertase in 69 potato varieties and lines amounted 9.33% on average. Totally 66 SNPs were identified (42 SNPs in exons and 24 SNPs in introns). Among the discovered individual nucleotide replacements 24 SNPs were localized in intron sequences, the intron polymorphism being greater than the exon one on the whole amounting 10.16%.

The exon sequences are especially interesting for study of allelic variants, because they code amino acids, and the changes in exons may eventually result in functional changes of protein. In the exon sequences being analyzed (exons 5 to 7) in 69 potato varieties and lines we have discovered 42 SNPs, which is 8.91% of the total length of studied exons. Such a degree of polymorphism in exons is unexpectedly high as compared with that detected in previous studies of vacuolar invertase gene. For example, during study of *Pain-1* homologue genes DNA in 219 potato specimens 28 individual point replacements have been described, 24 SNPs being detected in the analyzed fragment (exon 5–exon 7) [17]. In addition, we have previously studied polymorphism of this gene in a set of 19 Russian selection varieties and 25 SNPs have been identified on the said gene fragment of acidic vacuolar invertase at the total polymorphism degree of 3.53% [20].

The sequences of exons 5 to 7 of *Pain-1* were analyzed for presence of known [17, 20] and new nucleotide replacements, which have not been detected in other varieties yet. As a result, it appeared that among SNPs found in 69 studied potato varieties and lines specimens, SNP_{1544}, SNP_{1574}, SNP_{1596}, SNP_{1629}, SNP_{1661}, SNP_{1843}, SNP_{1857}, SNP_{1895}, SNP_{1896} (designated according to the sequential number of nucleotide on cDNA) have been described previously [17, 20]. In coding sequences of the specimens 34 nucleotide replacements

were identified; 25 of them have never been described previously: SNP_{1628}, SNP_{1648}, SNP_{1700}, SNP_{1709}, SNP_{1717}, SNP_{1724}, SNP_{1726},SNP_{1738}, SNP_{1788}, SNP_{1794}, SNP_{1797}, SNP_{1808}, SNP_{1815}, SNP_{1818}, SNP_{1831}, SNP_{1837}, SNP_{1847}, SNP_{1861}, SNP_{1865}, SNP_{1872}, SNP_{1885}, SNP_{1886}, SNP_{1890}, SNP_{1907}, SNP_{1909}.

It was reported that specific nucleotide replacements in the primary sequence of *Pain-1* can be associated with commercially valuable traits, such as increased starch content [17]. A significant replacement was found within the site in question, SNP_{1544} (C/A). It correlates with the increase in starch quantity in tubers and was detected in homozygous state in Ulan and Astana varieties. In Courtney, Courage, Elizabeth, Zhukovskii rannii, Ryabinushka, Favourite, Vector, Innovator, Kuznechanka varieties the SNP_{1544} replacement was also present, but in heterozygous state. Previously the presence of SNP_{1544} was demonstrated for one more variety of Russian selection —Bryanskii rannii [20].

The allelic variants of *Pain-1* gene fragments in potato varieties and lines. Totally in the analyzed set of 69 specimens of potato varieties and lines we have found 78 allelic variants of *Pain-1* acidic vacuolar invertase gene fragment (the data is presented in online version of the article on web site http://www.agrobiology.ru, Table 1). Herewith, 64 allelic variants appeared to be unique, 14 — shared by several specimens. The most widely spread allelic variants were Pain-1_A62 (in 13 specimens), Pain-1_A63, Pain-1_A64 (in 6 specimens), Pain-1_A12 (in 5 specimens). Each of the allelic variants Pain-1_A1, Pain-1_A28, Pain-1_A34, Pain-1_A36, Pain-1_A37, Pain-1_A38, Pain-1_A46, Pain-1_A65 was repeated in two specimens. For 15 varieties (Astana, Didar, Karassiyskii, Miras, Ushkonir, Ognivo, Lileya, Krepysh, Khozyayushka, Valentino, Nayada, Kamenskii, Lady Clair, Aroza, Spiridon) variety-specific allelic variants were identified, which are unique for the studied set.

The majority of 69 analyzed potato varieties and lines appeared to be homozygous, and several allelic variants corresponded to them. Varieties Ulan, Astana, Fritella, Ognivo, Lileya, Kholmogorskiyi, Krepysh, Khozyayushka, Valentino, Nayada, Kamenskii, Bryanskii delikatesny, Clair, Aroza, Didar, Karassiyskiy, Miras, Ushkonir, Spiridon and line 84 were homozygous in terms of the gene in question. The allelic variant Pain-1_A12 identified in varieties Fritella, Chernskii/1 and lines 84 and 111/1 appeared to be common with sequence *S. tuberosum* represented in database NCBI (GeneID:102577489) (see http://www.agrobiology.ru, Table 1).

Thus, in the fragment, being analyzed exon 5— exon 7 of *Pain-1* gene in 69 specimens of potato varieties and lines 78 allelic variants were identified (see http://www.agrobiology.ru, Table 1), which indicates high polymorphism degree. Previously, for full-size coding sequence of acidic vacuolar invertase only 11 allelic variants were described [17], and 9 allelic variants were found in 19 varieties of Russian, Byelorussian and Kazakhstan selection [20].

Amino acid sequences polymorphism analysis. Nucleotide sequences of exons were translated, and the supposed protein amino acid sequence was analyzed. Acidic vacuolar invertase consists of 639 aa. The length of the sequence available for analysis coded by fragment exon 5—stop codon was 156 aa, which corresponds to protein amino acid positions from 484 to 639. We selected the sequence of β-fructosidase of potato *S. tuberosum* from database NCBI GenBank (NP_001274993.1) as a reference sequence.

Totally 27 sites of amino acid replacements were identified in the sequences in question (the data is presented in online version of the article on web site http://www.agrobiology.ru, Table 2). Herewith, a characteristic of most specimens was the presence of singular amino acid replacements. However, in some specimens we found identical sets of amino acid replacements — uniform

amino acid patterns. For example, the common amino acid pattern was characteristic of eight potato varieties (Manifest, Arsena, Meteor, League, Breeze, Vesna belaya, Kolobok and Zolskii). One more common amino acid pattern was characteristic of varieties Aroza, Krepysh, Didar, Karasayskii, Miras and Kamenskii (see http://www.agrobiology.ru, Table 2).

The maximum quantity of amino acid replacements were discovered in Zhukovskii rannii variety (8 replacements) and lines 165 (8 replacements) and 162 (7 replacements). The overwhelming majority of specimens had 3 to 5 replacements. The absence of amino acid replacements as compared to the reference sequence was shown for Fritella variety and line 84 (see http://www.agrobiology.ru, Table 2).

The analyzed potato specimens were divided into three types: the first type is homozygous and, consequently, having one amino acid sequence variant (Ulan, Astana, Fritella, Ognivo, Lileya, Kholmogorskii, Krepysh, Khozyayushka, Valentino, Nayada, Kamenskiy, Bryanskii delikatesny, Lady Clair, Aroza, Didar, Karasayskii, Miras, Ushkonir, Spiridon, line 84); the second type is the specimens with heterozygous state of nucleotide sequences, in which the identified SNPs do not result in replacements of amino acid residues and change of amino acid sequence (varieties Bashkirskii and Ocharovaniye, lines 27 and 58); and finally, the third type — varieties and lines heterozygous in terms of nucleotide sequences, in which different gene alleles code non-identical amino acid sequences (remaining 34 varieties and 11 lines of potatoes).

Previously, during analysis of replacements for specific amino acid sites it was shown that only two variants of amino acid residues are absent [17]. In this paper, for the most part of identified polymorph sites the presence of only two amino acid variants is also characteristic. For example, for position 515 all studied varieties and lines have either residue of threonine (T), or residue of lysine (K) (see http://www.agrobiology.ru, Table 2). However, for two sites in amino acid protein sequence (amino acid residues 629 and 639) we found three replacement variants. For example, glycine (G) was in position 629 in varieties Red Scarlet, Yantar, Bashkirskii, Spiridon, Elizabeth, Zhukovskii rannii and line 9, serine (S) in Valentino variety, and alanine (A) in all other varieties and lines. In varieties Chernskii, Red Scarlet, Krasavchik, Kholmogorskii, Newton, Aladdin, Nadezhda, Favourite, Astana and lines 84, 58, 111, 152, 141, 7, 46, 162 the amino acid residue 632 in protein Pain-1 was arginine (R), in varieties Lileya, Ognivo, Yantar, Aroza, Krepysh, Didar, Karasayskii, Miras, Kamenskiy, Khozyayushka, Ushkonir, Courtney, Elizabeth, Courage, Ryabinushka, Vector — proline (P), in varieties Bryanskii delikatesnii, Ocharovaniye, Clair, Nayada, Bashkirskii, Spiridon, Lady Roseta and line 27 — glutamine (Q). In other specimens, the amino acid residues varied, depending on the allele (see http://www.agrobiology.ru, Table 2).

As noted above, SNP_{1544} replacement is the most important; it results in replacement of threonine with lysine (T515K). Herewith, the presence of lysine residue correlates with high starch content [17]. Among the analyzed specimens such replacement was discovered in varieties of Kazakhstan selection, Ulan and Astana, and in heterozygous state in varieties Kuznechanka, Sirenevy tuman, Courtney, Elizabeth, Courage, Innovator, Ryabinushka, Zhukovskii rannii, Vector and Favourite.

Thus, we have carried out the analysis of nucleotide and amino acid polymorphism of a gene fragment of acidic vacuolar invertase in 69 potato varieties and lines, which have never been studied on this attribute before. The presence of 42 SNPs has been demonstrated, including 34 sites described for the first time. The presented data witness a high degree of polymorphism of the gene

fragment in the studied specimens. A total of 78 allelic variants of *Pain-1* gene fragment have been discovered, which can further be used in selection for creation of varieties with increased starch content.

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LADY BEETLES FOR BIOCONTROL OF APHIDS, THE VECTORS OF VIRUSES, ON SEED POTATO PLANTS IN GREENHOUSES

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Abstract

Summer greenhouses are used for the production of seed-potato mini-tubers in many Russian regions, so developing technologies for the effective and environmentally friendly protection of seed potatoes against insects that are vectors of viruses in greenhouses is relevant for domestic potato growing. Predatory coccinellids (*Coleoptera*, *Coccinellidae*) are successfully used for aphids' control in greenhouses. However, these natural enemies have not yet been applied to protect seed-potato plants. This research is aimed at developing new approaches for applying *Harmonia axyridis* Pall. and *Cheilomenes sexmaculatus* Fabr. in the preventive biological control of aphids on seed-potato plants in greenhouses. Coccinellid larvae tolerance to fasting was evaluated in laboratory conditions at 24 °C and 60-70 % humidity. The optimal weight of the larvae to be preventively released into the greenhouses was determined. The IV instar larvae were kept with food in abundance for 1-2 days after molting. The larvae selected daily were grouped depending on their weight. The average time of starvation and the percentage of the individuals pupated were calculated for each size class of the larvae. To select indigenous coccinellids that can be used for aphid control on potatoes in greenhouses we monitored insects in natural habitats nearby the seed potato farm (Volosovskii Region, Leningrad Province). Monitoring of local entomophages and release of coccinellids were carried out in the seed potato farm. *H. axyridis* larvae were released in a film greenhouse (600 m^2) on the potato plants of cultivar Red Scarlett. The experiment continued from June 12 to July 10, 2017. The number of coccinellids (larvae, pupae, and adults) was estimated weekly. It was shown that even a 2-day starvation of the *H. axyridis* II-III instar larvae caused an 8-12 % decrease in the survival rate. Therefore, their use unfits for preventive colonization of potato plants. It is optimal to use the IV instar larvae if they weigh enough for pupation with the likelihood about 50 %. We recommend releasing *H. axyridis* larvae weighted 20-29 mg and *Ch. sexmaculatus* larvae weighted less than 9 mg. *H. axyridis* IV instar larvae found on the plants at the seed farm 7 days after releasing averaged 22 % of the total released number. A decrease in the number of *H. axyridis* larvae and mass pupation occurred 14 days after when pupated individuals were 9.3 % of the initial larvae number. The emergence of the *H. axyridis* imago was observed 21 days after the release. The obtained results prove there are some good reasons for application of the *H. axyridis* IV instar larva in greenhouses on seed potatoes. Monitoring of aphidophages in their natural habitats near the greenhouses allowed us to select local species of coccinellids which are promising for potato plant protection in greenhouses. In June-July 2017, potato aphids (*Macrosiphum euphorbiae* Thomas) appeared on nettle plants (*Urtica dioica* L.). *Adalia bipunctata* L. (imago and larvae) and *Coccinella septempunctata* L. (larvae) were found in the pest hotspots. *A. bipunctata* imagoes may be used in greenhouses because this species needs lower prey abundance for egg laying and consumes most aphids colonizing potato plants.

Keywords: biological control, *Coccinellidae*, *Harmonia axyridis*, *Cheilomenes sexmaculatus*, preventive colonization, resistance to food stress, cannibalism

The development of new environmentally friendly methods of protecting the seed potatoes is a new area in potato growing. Production of mini-tubers from test tube plants is one of stages of the original seed production. Summer greenhouses are used for their production in summertime in many regions of the Russian Federation, for example, Krasnodar Territory [1], North Ossetia [2],

Tatarstan [3], the Sverdlovsk Province (4), the Sakhalin region (5), and in the Leningrad Province. Growing mini-tubers in summer greatly increases the risk of attack of planting by insects, the carriers of viruses. The development of an efficient and environmentally friendly protection of seed potatoes against such insects in greenhouses of such type is challenging for the domestic potato growing industry.

The main strategy of protecting seed potatoes is preventive use of pesticides [3, 6]. It is necessary, because the hazard is not the damage caused by virus vectors (mainly, aphids), but the virus infection carried by them. Even test pricks of individual specimens of non-specialized aphid species can cause infection of potato plants by viruses [7]. When growing mini-tubers no infection of plants by viruses is allowed [8]. Consequently, the system of biological protection of seed potatoes shall also be based on preventive colonization of planting with entomophages.

Method of preventive application of entomophages in greenhouses has been developed for vegetable crops [9-11]. However, they require significant adjustment for use on seed potatoes. Growing virusless potatoes either precludes or restricts significantly the implementation of some standard techniques, which are usually applied for preventive colonization with aphidophages (for example, bringing accumulating plants with aphids into greenhouses). The aphid species for accumulating plants shall be selected depending on the nutritional adaptation of insects. The main requirement is that aphidophage shall not develop on the crop being grown. Greenbugs (*Metopolophium dirhodum* Walker, *Rhopalosiphum padi* L., *Schizaphis graminum* Rond., *Sitobiont avenae*Fabr.) or cabbage aphids (*Brevicoryn ebrassicae* L.) are often used [9, 11]. However, the said species often carry potato virus Y [12-14], due to what they are prohibited in case of seed planting.

Taking into account the limitations caused by peculiarities of cultivation of seed potatoes, it is necessary to compensate any losses and increase the strength margin of the protection system being developed, for example, by means of including new species of entomophages. In addition, it is necessary to adjust the methods of application of those entomophages, which are already used against aphids in the protected soil, but have not been approbated on potato crop yet.

A promising group of polyphagous entomophages is Coccinellidae (*Coleoptera*, *Coccinellidae*). Coccinellidae are widely used for protection of vegetable and decorative crops against aphids in greenhouses. Releases of younger larvae (or introduction of eggs), and releases of imago [9, 15-17] are used. The carrying of viruses by predatory Coccinellidae is almost impossible. They do not drink sap and oviposit onto the surface of leaves and culms without any damage of tissue. In addition, as distinct from specialist aphidophages (*Aphidiidae* and *Cecidomyiidae*), Coccinellidae exhibit an accelerated protective effect. Once released into greenhouses, Coccinellidae are capable of killing the victim (including virus carrying aphids) immediately, which precludes any possibility of the infection dissemination.

We have assessed the possibility of preventive colonization of seed potato planting with larvae of Coccinellidae *Harmonia axyridis* Pall. and *Cheilomenes sexmaculatus* Fabr for the first time. The basic criterion of screening of the species was stress resistance, which was defined as the time for which larvae preserved vitality when starving or absence of optimal feed. The approach we offered allowed selecting physiologically labile species capable of decreasing the intensity of metabolism in absence of feed faster than others. Species from various size classes at different development stages were tested, which allowed ob-

taining original data on the influence of the body weight and instar of larvae on their ability to survive on potato crop in the absence of the pest.

The goal of the research was the development of new methods of applying aphidophage Coccinellidae in the system of preventive protection of potatoes against aphids in greenhouses.

Techniques. The targets of the research were two species of predatory Coccinellidae — *Harmonia axyridis* Pall. and *Cheilomenes sexmaculatus* Fabr. The resistance of Coccinellidae larvae to starving was assessed in laboratory conditions at 24 °C and 60-70% humidity. Larvae of instars II and III were kept individually in Petri dishes and in groups of 5 bionts in 300 ml plastic containers without feed till pupation or death. In the control group, larvae were fed with greenbugs on cut wheat plants. The number of survived bionts, which molted into the next instar and pupated, was assessed daily.

For preventive releases of larvae into greenhouses, the optimal weight of bionts was determined. Larvae of instar IV were kept with the excess of feed (greenbugs or *Myzus persicae*) for 1 or 2 days after molting, and then bionts were selected for the experiment daily. The selected larvae were weighed on scales HTR-80CE (Shinko Denshi Co., Ltd, Japan), distributed over groups, depending on weight. The bionts in the experiment were kept individually without any feed until emergence of imago or death. In each of the separated larvae size classes, the average starving duration and the share of the pupated bionts were calculated.

Monitoring of local entomophages and release of Coccinellidae was performed in seed farm of Oktyabrskoye CJSC (Volosovskii Region, Leningrad). Larvae of Coccinellidae *H. axyridis* were released in a 600 m^2 film greenhouse onto Red Scarlett fluke potatoes. The test tube plants were planted on May 17, 2017 into 5 l vegetative pots (in total 8000 pcs). No pre-plant treatment of the root system with insecticides was carried out. The same-age larvae *H. axyridis* were used for releasing. They were grown from eggs collected within one day. The date of mass molting to instar IV was noted, and in 1 day larvae were selected for release into the greenhouse. The release was carried out from June 12 until July 10, 2017.

The quantity of Coccinellidae (larvae, chrysalides and imagoes) in the greenhouse was assessed daily. The accounts were performed by means of visual inspection of randomly chosen 1000 potato plants. Aphids and other pests were accounted simultaneously.

The weighted average (M) and the weighted average error (±SEM) were calculated using a package of statistical software Statistica10.0 (StatSoft Inc., USA). The difference confidence estimation was carried out using Student t-test. The differences were considered statistically significant at $p < 0.05$.

Results. Previously *H. axyridis* and *Ch. sexmaculatus* were selected as promising entomophages for protection in greenhouses based on high reproductive potential and trophic connections with aphids, which are common in potato agrocenosis [18]. In addition, they are found on potatoes on the field [19, 20]. For protection of vegetable and flower crops against aphids in greenhouses, larvae *Harmonia dimidiata* Fabr., *Coleomegilla maculata* Degeer, *Coccinella septempunctata* L., *Cycloneda limbifer* Casey, *Adalia bipunctata* L., *Propylea quatuordecimpunctata* L., *P. japonica* Thunb. are also used. It is recommended to release mainly larvae of ages I-II to the pest concentration centers. If the food is available, larvae complete their development and pupate in greenhouses. Duration of the protective effect of the release is 7 to 14 days, depending on temperature and species of Coccinellidae [9, 16, 21-23]. The protective activities on potato plants shall ensure the total absence of aphids in the greenhouses. Therefore, the strate-

gy of releasing younger larvae in expectation of further development of Coccinellidae due to eating phytophages cannot be implemented. In the absence of the pest, larvae *H. axyridis* of ages II-III perish without food within 2 days. Even if a larva finds a victim after 2 day starving, it cannot compensate its loss of weight and vitality. The compensatory growth of larvae after short-term starving is only possible at instar IV [24].

According to our data obtained as a result of mass and individual growing of *H. axyridis*, 2-day deficiency of feed for larvae results in irreversible adverse effects, which was expressed in decrease of larvae which molted to instar IV down to 8-12% at individual growing. In case of mass growing this index used to rise to 20-23%, evidently due to cannibalism (Table).

Survival capacity of larvae *Harmonia axyridis* Pall. after2-day starving (laboratory test)

Age	Content	Variant	Quantity, pcs	Molted to instar IV		Pupated	
				total, pcs	*M*±SEM, %	total, pcs	*M*±SEM, %
II	Individual	E	50	4	8±1.0*	1	2±0.3*
		C	50	35	70±3.0	32	64±3.3
II	Mass	E	100	20	20±1.6**	6	6±0.6*
		C	100	65	65±2.3	58	58±2.4
III	Individual	E	60	7	12±1.3**	0	0*
		C	50	39	78±2.4	27	54±3.5
III	Mass	E	100	23	23±1.8**	11	11±1.0**
		C	120	84	70±1.9	58	48±2.3

Note. E and C stand for experiment and control groups respectively (the feed in the control group is greenbugs).
*, ** Differences with control group are statistically significant respectively at $p < 0.001$ and $p < 0.01$.

Consequently, it is not expedient to release larvae of Coccinellidae aged II-III on potatoes for preventive colonization. It is necessary to seek other approaches in their use. One of possible solutions is releasing instar IV larvae, which are capable of compensating losses caused by temporary limitation of food resource. It is necessary to note that at instar IV larvae of Coccinellidae are highly gluttonous. They eat about 70% of the total amount of aphid consumed during larval development. Therefore, their potential as entomophages at instar IV is much higher than at all preceding ages combined [15, 21-23]. At the preventive colonization, the efficiency of releasing the larvae ages IV will depend on their resistance to nutritional stress (absence of feed) which is determined by the duration of starving before pupation or death.

Based on the results of laboratory testing, the optimum is release of larvae *H. axyridis* weighing 20-29 mg and larvae *Ch. sexmaculatus* weighing < 9 mg, because in these variants of the experiment the larvae starving duration was maximum ($p < 0.05$). In particular, on day 4 in these size classes 30 to 40 % of larvae showed delay of metamorphosis and preservation of motor activity. In other variants of the experiment on the 4th day of starving the majority of larvae used to pupate (Fig. 1). In the selected size classes 42% of larvae *C. sexmaculatus* and 60 % of *H. axyridis* pupated. Consequently, potentially about a half of the released bionts were able to develop to imago stage and with breed. In the environment of age-synchronised crop, the larvae achieved the optimal weight 1 or 2 days after molting to instar IV, depending on the species.

The results obtained during laboratory testing of *H. axyridis*, witness the prospectivity of using instar IV larvae in greenhouses for the purpose of protecting the seed potatoes against aphids. For estimate of *Ch. sexmaculatus* further studies of trophic connections are required. So far, there is no data if the predator feeds on *Macrosiphum euphorbiae* Thomas, the potato virus Y carrier [18]. Direct evidence is required that this entomophage can liquidate *M. euphorbiae*, especially taking into account relatively small size of larvae from the size class

which is optimal for release (less than 9 mg).

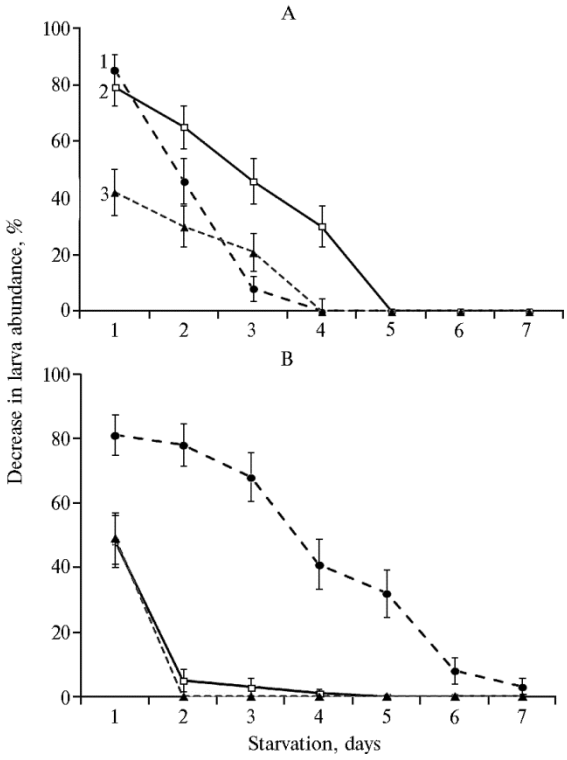

Fig. 1. Dynamics of decreasing quantity of instar IV larvae *Harmonia axyridis* (A) и *Cheilomenes sexmaculatus* (B) (as a result of pupation or death) in relation to the initial quantity during starvation: 1 — weight of larvae < 19 mg (A: n = 39; B: n = 73), 2 — 20-29 mg (A: n = 105; B: n = 328), 3 — 30-39 mg (A: n = 121; B: n = 97) (laboratory test).

Laboratory experiments for assessment of resistance of Coccinellidae larvae to nutritional stress were carried out at 24 °C which was optimal for larvae development. As temperature decreases, the larvae vitality preservation time shall increase. This supposition was confirmed in production tests. Instar IV larvae of *H. axyridis* were released in the greenhouse at mean daily temperature 18-21 °C. From June 12 until July 10, 2017 680 larvae were released onto 3400 plants.

Individual *Macrosiphum euphorbiae* were found on potatoes, mostly winged disseminating females. The share of plants colonized by aphids did not exceed 0.1 % (7-8 plants per greenhouse). Despite the almost total absence of the pest, 30.4% of the released *H. axyridis* were detected on the plants 7 days after the release. The share of larvae amounted 21.8% of the initial value (Fig. 2). In 14 days a confident ($p < 0.05$) decrease of number of larvae, *H. axyridis* and mass pupation was observed. A total of 9.3% of the initial number of larvae were found as pupated bionts on plants. Emergence of imago *H. axyridis* was noticed 21 days after the release of larvae.

The share of larvae, which remained on the plants in our experiments, was on average comparable or more than in the traditional method of colonization (release of younger larvae to the pest centers). For example, after instar I larvae *H. axyridis* release to greenhouses onto hautbois plants, 22.0% of the initial number of Coccinellidae were found 7 days later [25]; 7.5-12.5% of bionts were found on herbaceous crops 4 days after the release of instar I-II larvae *H. axyridis* [26]. Similar results have been obtained when other Coccinellidae species larvae were released. For example, 8 days after the release of instar I larvae *A. bipunctata* and *C. septempunctata* 19.0% and 1.3% of the initial number of Coccinellidae were found respectively [27]. It means that as early as 4 to 8 days after release of younger larvae their number decreases significantly (by 80-90%), despite there are aphids in agrocenosis. It is evident that in the absence of victim the decrease of the number of released larvae will be even faster.

A possible environmental and genetic mechanism ensuring the efficiency of our suggested variant of preventive colonization of *H. axyridis* was heterogeneity of physiological response of this species to nutritional stress (absence of aphids). The population contained some share of instar IV larvae which delayed metamorphosis, despite the quantity of the nutrients accumulated by them ena-

bled their pupation. It can be supposed that such larvae remained on the plant and contributed into the protective effect of the preventive colonization.

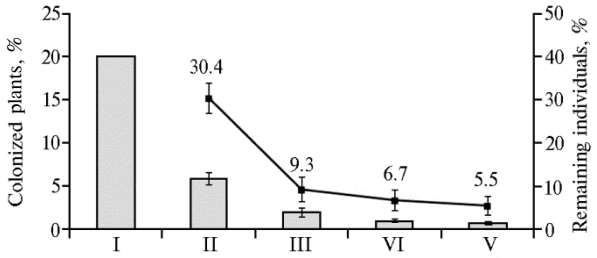

Fig. 2. The share of colonized plants (diagram) **and *Harmonia axyridis* bionts remained on them** (plot) **after release of instar IV larvae:** I — release (larvae), II — day 7 (71% of larvae, 29 % of chrysalides), III — day 14 (chrysalides), VI — day 21 (imago), VII — day 28 (imago) (Oktyabrskoye CJSC, Leningrad Province, June-July, 2017).

However, the leading role in the preventive release of *H. axyridis* was evidently played by instar IV larvae, which did not reach the threshold weight necessary for metamorphosis. In the laboratory experiments in individual maintenance without feed, the larvae with weight deficit preserved motor activity 3 to 4 times as long as the bionts which have already reached the pupation threshold. Among the larvae released by us to the greenhouse (20-29 mg) the share of bionts with subthreshold weight was significant (about 50%). To survive in the greenhouse, they were to find food. Such food could be aphids or bionts of the same species (larva, chrysalide, young imago).

Cannibalism is characteristic of Harmonia larvae. Eating bionts of its species is an integral part of the behavior pattern of *H. axyridis* [28]. Possibly, it was cannibalism that enabled surviving in the greenhouse for larvae with subthreshold weight. When using the larvae from our recommended size class (20-29 mg) the supposed losses due to cannibalism will be partially compensated with decrease of mortality among the subthreshold weight larvae.

Releasing instar IV larvae *H. axyridis*, we noticed the accumulation of entomophage in agrocenosis at pupal stage. If younger larvae are used, then their pupation without feed in the greenhouse is unlikely. For example, in case of paucity of aphids on chrysanthemums in the greenhouse 0.6% of the total released instar I larvae *A. bipunctata* pupated, and when larvae *C. septempunctata* were released, no chrysalides were found [27]. Concerning the quantity of *H. axyridis* chrysalides in the greenhouse, one should allow for the fact that the accounts were on the plants only. Possibly, some part of released larvae pupated outside the plants: on the walls of vegetative pots, on the film covering the greenhouse floor, etc. It is highly probable that some chrysalides were eaten by larvae of the same species.

In addition to well spread cannibalism, *H. axyridis* has one more species feature, which should theoretically prevent from accumulation of entomophages in the greenhouse. In the lifecycle of *H. axyridis* there is a mandatory period of migration state, which follows right after the emergence from chrysalide and ensures dispersal of the new generation bugs from hatchings [15]. During colonization in the greenhouse, one can forecast migration of a part of young imago to territories near the greenhouse. However, as applied to conditions of Oktyabrskaya CJSC, we assess the migration of *H. axyridis* as a positive phenomenon, because the Coccinellidae, which flew away, could control the pest on potato plantings on the field just near the greenhouses. In addition, Coccinellidae imagoes, which migrated to territories near the greenhouse, will strengthen the natural populations of entomophages, which restrict the development of aphids in natural stations around the greenhouses.

As a whole, it can be concluded that the greenhouse planting of virusless potatoes is an agrocenosis the conditions in which facilitate seasonal coloniza-

tion of Coccinellidae. Thanks to long-term crop vegetation time (3 to 4 months), availability of safe pupation locations and absence of active manipulations with plants, it becomes possible to accumulate the released ladybirds.

For reference, on lettuce lines the seasonal Coccinellidae colonization failed despite high release rates [9]. Lettuce crop has short vegetation time (30 days on average). Entomophages with finished products are removed from the greenhouses. The emptied hydroponic bed installations are disinfected as plants are removed, destroying the predators pupated on them. Due to permanent movements of boxes with plants, the ladybird larvae may fall under the beds. Since the potato planting in soil greenhouses are free from the above listed drawbacks, we consider the application of Coccinellidae larvae in these agrocenoses to be very promising.

Taking stock of our production tests, we can issue the preliminary recommendations concerning the frequency of releases of *H. axyridis* for protection of seed potatoes in the greenhouses. Since 1 week after the release only 22% of *H. axyridis* remained at the larva stage, the releases should be carried out at least once per 7 days, so that the larvae ensuring the protective effect were permanently present on the plants. Further research is necessary to determine the optimal release rates. Herewith, it should be taken into account that the excessive increase of the density of ladybirds on the plant is not expedient due to high risk of larvae cannibalism in the absence of aphids, as it was noted in the experiments with *H. dimidiata* on cucumber crop in the greenhouse [22]. However, there is a risk of the insufficient Coccinellidae larvae release rates resulting in the protection system weakening, because not all plants will be inspected by the larvae. To enhance the protective effect reliability, it is expedient to consider a variant of the combined application of ladybird larvae with other aphidophages. The data has been obtained that Aphidiidae avoid the plants with traces of Coccinellidae larvae and imago for 24 hours [29]. Consequently, the repellent effect caused on Aphidiidae by ladybirds makes the parasites to inspect mostly those plants, which have not been visited by predators. It can be supposed that the combined use of the predator and parasite will facilitate the continuous protection of the maximum amount of plants. The ladybird imagoes exhibit high search activity and are characterized by significant life duration (no less than 20 to 30 days for small-size species and 2 to 3 months for big-size ones). Due to such features, the ladybird imagoes are promising for protection of potatoes in the greenhouses and on the territory near the greenhouse.

To select Coccinellidae suitable for protection of potatoes in the greenhouses, a monitoring was carried out. It covered the local entomophage species, which live in natural stations near the greenhouse system of Oktyabrskoye CJSC. In June and July, 2017 *Macrosiphum euphorbiae* was discovered on nettle (*Urticadioica* L.) plants. In the pest centers Aphidiidae (aphid mummies), *A. bipunctata* (imago and larvae) and *septempunctata* (larvae) were noticed. It should be noticed that *Macrosiphum euphorbiae* form rarefied colonies. It should be taken into account during screening of entomophages suitable for pest control. It is expedient to select the ladybird species for females of which individual aphid bionts are sufficient for oviposition. One of such species is Adalia [30].

The ladybirds' ability to lay eggs on the background of low number of aphids is in negative correlation with the imago weight [31]. *A. bipunctata* is included into the small-size class, imago weight is 10-18 mg). Herewith, the following aphid species commonly found in potato argocenoses were noticed among victims of *A. bipunctata*: *Acyrthosiphon pisum* Harris, *Aphis fabae* Scopoli, *Aphis gossypii* Glover, *Aphis nasturtii* Kalten., *Aulacorthum solani* Kalten, *Brachycaudus heli-*

chrysi Kalten, *M. euphorbiae*, *Myzus persicae* Sulzer, *Rh. padi* [18].

In addition to the variety of victims and low oviposition induction threshold, there is one more argument in favor of using Adalia or similar-sized species on potatoes. During preventive colonization, not high gluttony (characteristic of big ladybirds) is required from the predator, but the ability to find and destroy the primary pest center. Herewith, the cost of growing one biont of small-sized Coccinellidae species is 2 to 3 times as cheap as that of big-sized ladybirds. Therefore, it is economically more expedient to release imagoes of small-sized Coccinellidae species. Besides, in case of migration from the greenhouse it is more probable that such ladybirds will stay on the territories near the greenhouses, eating the pests, which are regularly brought by the wind from natural stations and personal subsidiary plots.

Concerning the prospects of applying *C. septempunctata*, the big size of the ladybird should be taken into account (imago weight is 25-50 mg), due to which is needs high aphid density for development of the breed. The *C. septempunctata* oviposition is induced by aphid colonies consisting of dozens bionts, and for the development of the next generation over 100 bionts are necessary [32]. The emergence of such unfavorable phytosanitary situation is unlikely on the territories near the greenhouse close to seed potatoes planting. Evidently, insecticides will be applied against aphids in case of a risk of pest outbreak. Therefore, we think that it is inexpedient to include *C. septempunctata* imago into the complex of entomophages for preventive releases. However, it should be taken into account that the said species is one of dominating predators in potato agrocenoses on the field [19, 33, 34]. Herewith, *C. septempunctata* is known as a wide polyphage, and in the absence of aphids, it can feed on arthopods, and plant pollen and fungal spores [35]. The dominating position of *C. septempunctata* among natural predators on potatoes and the wide nutritional adaptation allow recommending the activities for preservation of its wintering areas near greenhouse seed potatoes production complexes. High population numbers of this entomophage will facilitate enhancement of phytosanitary situation as a whole.

Thus, based on the results of the conducted screening of Coccinellidae aphidophages for protection of seed potatoes, we selected *Harmonia axyridis* Pall species, which we recommend to release at larval stage, 1 or 2 days at instar IV. For the guaranteed protective effect, the *H. axyridis* larvae releases should be carried out at least once per 7 days. The natural populations of *C. septempunctata* and *A. bipunctata* for protection of seed potatoes is possible if a technological solution is found which would allow to purposefully attract these entomophages to the territories near greenhouses, for example, by planting nectariferous plants. As a whole, the technology of cultivation of virusless potatoes allows accumulating the released ladybirds, so the application of Coccinellidae larvae in such agrocenoses is highly promising.

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TO THE EXPERIMENTAL CONFIRMATION OF THE HYPOTHESIS ABOUT AN ECO-GENETIC NATURE OF THE PHENOMENON GENOTYPE × ENVIRONMENT INTERACTION FOR WOODY PLANTS

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Abstract

The hypothesis of the eco-genetic nature of the phenomenon genotype × environment interaction (GEI) was developed by Russian scientists in 1984 after discovering an epigenetic phenomenon, the change in the spectra of genes that determine the same quantitative trait in annual crops with a change in the limiting factor. Perennial fruit trees are ideal objects for studying mechanisms of genotype × environment interaction. Genetic diversity within a fruit tree variety is practically zero as the trees are genetically homogeneous clones due to grafting, plants in a commercial garden are of the same age because of simultaneous planting, and they have the same soil area of nutrition, that is, there is no superposition of genetic and environmental competition. Annual growth in thickness of trunk and branches is very informative, and the pattern of a tree's annual growth rings records information about growth conditions, above all weather conditions, for many years. We studied apple (*Malus domestica* Borkh.) and apricot (*Prunus armeniaca* L.) varieties of northern and southern origin which possess different tolerance to weather stressors. To reveal alterations in the spectra of genes determining an increase in the thickness of a tree trunk and branches, we compared annual rings in the commercially grown trees of different adaptiveness which undergone the action of various limiting weather factors changing over a long period, particularly the effects of dry and hot years in contrast to the wet and cool years. It was revealed that wet and cold weather caused a bigger increase in branch thickness in the northern-originated apple variety Krasa Severa from Ekaterinburg with better genetic and physiological systems for cold resistance, whereas dry and hot weather similarly affected the southern-originated variety Bahorn from Uzbekistan. Pubescence of leaves and cuticle thickness which contribute to drought resistance cannot contribute to an increase in cold resistance. Hence, a change in the incremental thickness grades suggests a change in the set of component traits, and, therefore, the sets of genes that determine these traits, with a change in the limiting factors of the environment. Thus, due to the choice of the varieties of different origin and adaptability and the years with contrasting limiting environmental factors, we succeeded to discover the facts that were predicted by the hypothesis of the nature of the genotype × environment interaction phenomenon.

Keywords: nature of genotype × environment interaction, fruit crops, adaptability, change of gene spectra

In 1984, a group of executives of the cooperative programme DIAS (Genetics of production character of summer wheats of Western Siberia) discovered a new epigenetic phenomenon — changing the set of products of genes determining the same polygenic character. It has been established that productivity and yield are determined by changing sets and number of gene products at the

change of the environmental limiting factors]1].

The concept of genotype × environment interaction (GEI) in a narrow sense appeared at the start of ecological tests of pre-varieties and varieties. The phenomenon of changing productivity ranks in a set of varieties was identified, when they were tested in different years in the same geographical location, or in the same year, but in different locations. The GEI phenomenon appears if there are at least two genotypes, and it is expressed in the change of their ranks on production characters at least in two environments [3, 4]. If in one econiche the ranks of four varieties in terms of productivity are 1, 2, 3, 4, and in the other 1, 2, 3, 4, then GEI is equal to zero. If in one environment the ranks are 1, 2, 3, 4, and in the other 4, 1, 3, 2, then GEI is present. When the same genotype is studied in different environments (comfortable and uncomfortable), then GEI phenomenon cannot be observed, because only non-heritable changes of characters (modifications) will appear [5]. Y.N. Sinskaya [6], and later S.G. Inge-Vechtomov [7] noted that the deficit of knowledge about the nature of modifications remains a significant problem in genetics. The mechanisms of modifications are being studied intensely now [8-12], but there is no complete understanding of their nature so far. GEI is a more complex phenomenon than modification and, possibly, this is why there was not any hypothesis on GEI nature until the end of the 20th century either in classical genetics (Mendelism), or in biometrics and modern molecular genetics.

At the beginning of the 20th century, K. Pierson [13] formulated a rigorous definition for GEI phenomenon and suggested a rank correlation coefficient for its measurement. Later R.A. Fisher [14] created two-way analysis of variance for a more rigorous quantitative estimate of GEI effects. Now, there are different statistical methods for quantitative determination of GEI effects [15-20]. However, their estimate in different environments without understanding of the nature (mechanisms) of this phenomenon does not allow building a system of forecasts for values and shifts of GEI effects, which does not allow predicting productivity ranks and yields of a specific variety in different geographical locations.

The hypothesis of the GEI nature evolved from the theory of ecological and genetic organisation of polygenic characters (TEGOPC) was formulated as follows: “Mechanism of GEI effects is the change of set of gene products available in cells of varieties and determining the production character at the change of the environment lim factor”. The hypothesis was checked on the character “transpiration rate” (TR) in two groups of varieties of summer wheat: group I with large, closely placed stoma on leaves and thick, dense cuticle, and group II with small, widely-spaced stoma and thin, spongious cuticle. The morning TR was more intense in group I varieties, the day (cuticular) TR was more intense in group II. In the morning, the TR differences between groups of varieties were determined by genetic systems checking the size and spacing of stoma on the leave, at noon — by wax synthesis genes determining the cuticle thickness and density. Herewith, the TR ranks of groups changed, i.e. the GEI effect appeared. Its mechanism is evident: the change of sets of gene products determining the TR character [22].

When solving the vertical inheritance problems, one should select objects with short onthogenesis period (drosophila, arabidopsis, coliform bacterium). To decrypt the GEI phenomenon mechanisms (the problem of “heritable implementation”), due to absolute lack of knowledge of the paths from GEI to a particular gene [23], we selected an ideal, as we deem, object (perennial fruit plants) and an ideal character (the thickness of annual wood increment in the required year).

The perennial fruit plants have some advantages for research into GEI mechanisms. They propagate by graftage, i.e. each variety is a clone, inside which the genetic diversity on character determination is actually equal to zero. The orchards of perennial fruit trees are planted simultaneously with same-age plantlets, i.e. all the trees are the same age. The trees are planted equispaced, they have equal growing spaces (there is no genetic and environmental competition). To increase the yields, several varieties are planted in the orchard, so that intercross would occur due to pollen of different genotypes; it precludes any diminishing of yield due to self-fertilization. Many varieties of fruit trees have been created all over the world (from northern to southern), so one can always find varieties different in terms of adaptability in the same orchard. The character "annual growth ring area" in the required year (on the trunk or branch saw cut) enables estimating the averaged quality of growth conditions in each year for a long-term period. This character logs information about integral conditions of the year, which is thus preserved for the whole life of the tree.

In this paper, the hypothesis of ecological and genetic nature of GEI phenomenon is experimentally checked for the first time on an ideal object, the varieties of perennial fruit crops.

The objective of the research was decryption of epigenetic nature of "genotype × environment interaction" (GEI) by experimental verification of the GEI nature hypothesis.

Techniques. The research was carried out in K.A. Timiryazev Experimental Production Farm (the Krasnodar Territory, Ust-Labinsk city), Tsentralnoye Experimental Production Farm CJSC, FSBSI North Caucasian Federal Scientific Centre for Horticulture, Grape Culture, Wine-Making and Plodovod LLC (Krasnodar city). It included the study of varieties with different degree of adaptability depending on their origin: apple tree varieties (*Malus domestica* Borkh.) Bakhorn from Uzbekistan and Krasa Severa from Yekaterinburg (Tsentralnoye Experimental Production Farm CJSC), apricot variety (*Prunus armeniaca* L.) New Jersey from USA and local variety Krasoschyokii (K.A. Timiryazev Experimental Production Farm LLC, Plodovod LLC).

The annual wood thickness increment rank change was analyzed in three surfaces from three trees according to methodology of G.N. Terenko [20]. The thickness was measured on cores extracted from the main branches or on branch saw cuts using a drill. In the weather database the years with different combination of temperature and humidity regime were selected (humid and cold; dry and hot), that is those, in which the annual increment thickness ranks change and GEI phenomenon manifestation were expected. The average growth ring width and the ring area were measured for the required year.

During statistical data processing, the average values of the annual growth ring width or area (M) and errors of mean ($\pm$SEM) were calculated.

Results. The hypothesis postulates that the GEI nature is the change of gene products determining the same character at change of the environment limiting factor [21, 22, 25]. In a dry hot year, the annual wood increment thickness shall be greater in a southern drought resistant variety, because it carries the best genetic and physiological systems of drought and heat resistance formed by natural and artificial selection over the years of the variety breeding in dry hot zone. In a humid and cold year, the annual growth thickness shall be greater in a variety created in a moderate or northern zone and having better cold-resistant systems.

The obtained data confirmed validity of the formulated hypothesis (Table 1, 2). In a humid and cols year, the annual branch thickness increment shall be greater in the northern apple tree variety Krasa Severa, in a dry and hot year — in southern variety Bakhorn.

Since such component characters contributing into the drought resistance enhancement as pubescence of leaves and cuticle thickness cannot contribute into the cold resistance in principle, it means that the change of ranks on the annual wood growth thickness indicates to the change of the set of adaptability component characters and the gene products sets determining these characters at the change of the environment lim factor.

1. The annual primary branch grown thickness in two apple tree varieties (*M*±SEM, orchard planted in 2007, rootstock M 9, Tsentralnoye Experimental Production Farm CJSC, Krasnodar city)

Variety	Origin	Average annual growth ring thickness, mm		Annual growth ring area, mm^2	
		2011 (HC)	2012 (DH)	2011 (HC)	2012 (DH)
Bakhorn (n = 9)	Uzbekistan	0.78±0.19	0.95±0.18[a]	1.91±0.09	2.83±0.07
Krasa Severa (n = 9)	Sverdlovsk	1.50±0.46	0.75±0.12[a]	7.07±0.22	1.77±0.11

Note. HC — humid and cold year, DH — dry and hot year. Statistically significant at significance level 5%. Between the variants marked with letter ([a]) there are no statistically significant differences at p = 0.05.

2. The annual primary branch grown thickness in two apricot tree varieties (*M*± SEM, orchards planted in 2006, rootstock: cultivar plantlets, wild apricots)

Variety	Annual growth ring area, mm^2		
	2007	2010	2014
Plodovod LLC (Krasnodar city)			
New Jersey (n = 9)	27.45±0.77	196.37±0.98	341.12±7.00
Krasnoschyokiy (n = 9)	23.12±0.84	119.94±0.88	428.50±1.42
K.A. Timiryazev Experimental Production Farm LLC (Ust-Labinsk city))			
New Jersey (n = 9)	29.00±2.53	185.00±3.81	697.34±4.72
Krasnoschyokii (n = 9)	13.52±0.13	73.30±0.34	498.40±1.12

Note. VDH — very dry and hot yea; LDH — less dry and hot year, WI — winter icing (there was no icing in K.A. Timiryazev Experimental Production Farm LLC in 2014).

Summer 2007 in Krasnodar city and Ust-Labinsk city was very hot and dry, summer 2010 was characterized also by high air temperatures on the background of long-term moderate drought. Herewith, conditions in Ust-Labinsk city were significantly severer in terms of water availability and increased temperature regime in summer vegetation period.

The annual growth ring areas in both inspected apricot species appeared to be much greater in 2010, which means more comfortable growth conditions (see Table 2). The significant differences in the annual growth ring areas of primary branches of Krasnoschyokii variety trees in 2007 and 2010 were caused by the variety's response in yield, which in this case ensured a more intense growth of young apricot trees.

In 2014 from January 22 until 24, an abnormal weather phenomenon was observed in Krasnodar city – ice rain (precipitation in the form of a rain at air temperature 0 or –1...–2 °C). It resulted in gradual icing of trees, which remained in such a state for 2 days. The icing caused significant damages of vegetative organs and reproductive buds of all fruit cultures (especially apricots) and, consequently, inhibition of growth. There was no such a phenomenon in Ust-Labinsk city. In Ust-Labinsk city, the variety New Jersey exceeded the variety Krasnoschyokii in terms of the annual growth thickness in all three years, and lim factor "ice rain" in Krasnodar city resulted in the change of ranks of varieties, i.e. to expressed manifestation of GEI phenomenon (see Table 2).

Thus, by means of selecting the years contrast in terms of lim factors from the weather data bank and by selecting varieties different in terms of origin and adaptability, it became possible to discover the facts, which were predicted by the hypothesis of "genotype × environment interaction" (GEI) nature. The ex-

perimentally validated hypothesis of the nature (mechanisms) of GEI phenomenon allows forecasting GEI phenomena in a new environment, if typical behavior of lim factors is known for it. Knowing the geographical locations of origin of the varieties used for the experiment and the main lim factors in such locations and comparing them to typical behavior of lim factors in the new environment where we want to introduce our varieties, one can forecast the varieties' ranks on yields before carrying out of the experimental introduction. It opens perspectives of a new approach to selection of varieties introduced into other zones and allows assessing specific genetic drawbacks of each variety for their subsequent elimination using selection methods for the complete compliance of each new variety with typical lim factors of the growing zone.

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PHYSIOLOGICAL AND GENETIC COMPONENTS OF BLACK ROT RESISTANCE IN DOUBLE HAPLOID LINES OF *Brassica rapa* L.

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Abstract

In some vegetation seasons black rot may damage up to 80 % of cabbage, turnip, rapeseed, mustard crop all over the world including Russia. To prevent the spread of black rot is difficult, and it is almost impossible to fight a pathogen penetrating into a susceptible plant. Among *Brassica rapa* L., the disease is most dangerous for root crops and leaf crops. Data on loci which determine the plant-specific resistance of *B. rapa* plants to black rot is still extremely limited. This study is the first to estimate resistance to four races of *Xanthomonas campestris* pv. *campestris* (Pam.) Dow., the causative agent of black rot in *Brassicaceae*, in the lines of doubled haploids of two *B. rapa* mapping populations, DH38 (♀P175 × ♂P143) and DH30 (♀P115 × ♂P143). Here, we report data on identification and mapping the linkage groups and QTLs associated with physiological resistance to strains PHW231 (race 1), HRI5212 (race 3), HRI1279a (race 4), and B-32 (race 6). For three of these races, OTLs have not been mapped so far. The study revealed lines which were resistant or hypersensitive to the four races of black rot agent. Monogenic non-linked inheritance of resistance to these races prevailed. Significant correlation was found between response to an individual strain and general infection in plants. A total of 13 QTLs which control resistance to four races of the black rot pathogen were identified for DH30 population and 19 QTLs were found for DH38 population. All detected loci did not change their localization during two years of investigation. The most important loci responsible for manifestation of physiology resistance to different races of black rot pathogen in DH30 were mapped in the linage groups A01, A03 and A07, whereas in DH38 these were in A03, A06 and A08. SSR analysis of the lines contrast in resistance to individual races of the pathogen revealed the microsatellite markers linked to the loci which control resistance to several races of black rot agent. So we have found effective molecular descriptors of *B. rapa* black rot resistance to each race separately and to the pathogen as such. The obtained data are of interest in elucidation of basic physiological and genetic mechanisms of gene-to-gene interaction and *B. rapa* resistance to different races of *X. campestris* pv. *campestris*.

Keywords: *Brassica rapa* L., *Xanthomonas campestris* pv. *campestris* (Pam.) Dow., black rot resistance, QTL mapping, SSR markers, molecular screening

Vascular bacteriosis is the most harmful and widespread bacterial disease of plants of the *Brassicaceae* family in the world [1, 2]. In some years, it affects up to 80% of the crops of cabbage, turnip, rape, mustard, including in Russia. It is difficult to prevent the spread of vascular bacteriosis, and the only means is to use disinfected seed and to eliminate potential sources of

infection in the field [3]. Fighting the pathogen that has penetrated a susceptible plant is almost impossible. Vascular bacteriosis in cabbage is caused by pathovar *Xanthomonas campestris* pv. *campestris* (Pam.) Dow. (hereinafter *Xcc*), which includes a large number of races [4]. The pathogen penetrates the plant through hydathodes, stomata and mechanical damage, and colonizes the xylem. Symptoms of vascular bacteriosis include edge chlorosis on the leaves, necrosis and darkening of the leaf veins and conductive tissues inside the stem. In the species *Brassica rapa* L., the disease is most dangerous for turnip root crops and leaf crops [5], including the widespread Chinese cabbage [6].

The *B. rapa* species combines important oilseeds, vegetables and feed crops, and serves as a model object for genetic and molecular research. Despite the high importance of this species for human nutrition, there are few publications on the genetic nature and inheritance of morphological, physiological, immunological and other economically valuable traits in *B. rapa*. For example, the geographical and taxonomic distribution of race-specific resistance to phytopathogenic xantomonads in samples of *B. rapa* and *B. napus* was studied [7]. A high frequency of resistance to race 4 was found among the *B. rapa* subspecies of Central Asian and Japanese origin and in *B. napus*. For the first time, donors were found of the complex resistance of *B. rapa* to all used races of the pathogen and sources of resistance to races 1 and 3 among the samples of *B. rapa* and *B. napus*. QTL (quantitative trait loci) identification using RFLP (restriction fragment polymorphism) and RAPD (random amplified polymorphic DNA) markers showed that the plant response is associated with several additive loci in different linkage groups [8]. The *Xcc* gene of resistance to race 4 in Chinese cabbage (*B. rapa*) is located about 3 cM from the clubroot resistance locus [9]. Inheritance of resistance to three *Xcc* races was studied using hybrids from crossing resistant and susceptible *B. oleracea*, *B. carinata* and *B. napus* lines [10]. A single dominant *Xca4* locus (resistance to race 4) was mapped in the doubled haploid lines that were used to create an RFLP map. The *Xca4* locus was located in the N5 linkage group of the A genome in the *B. napus* species, thereby confirming that resistance was inherited from *B. rapa*. *Xca4* was the first mapped main locus controlling race-specific resistance to *Xcc* in *Brassica* species. British and Spanish scientists [11] studied the inheritance of resistance to two the most common *Xcc* races 1 and 4 in the segregating generation F_2 from crossing a sample of Chinese cabbage B162 with non-specific resistance and a susceptible inbred R-o-18 line. They created a genetic linkage map with a total length of 664 cM based on 223 AFLP (amplified fragment length polymorphism) and 23 microsatellite markers. Resistance to both races correlated. The authors localized a cluster of highly significant resistance QTLs determining 24-64% of the trait variability in A06 linkage group. Two additional minor resistance QTLs to race 4 were found in A02 and A09 linkage groups. Earlier, we studied two mapping populations of *B. rapa* in order to identify QTLs that determine morphological and phenological characteristics [12-15]. QTL positions have been identified (mainly in A02, A03, A06, A07 and A09 linkage groups), where one locus controls several traits, which indicates the important role of this locus in plant development.

At the same time, there is still very little information on the location of loci that determine the race-specific resistance of *B. rapa* plants to vascular bacteriosis. This study is the first identification i) of chromosomal loci that control resistance to the four most common races of the causative agent of vascular bacteriosis in doubled haploid lines of two *B. rapa* mapping populations, and ii) of new resistance donors found in these lines.

Our goal was to identify and map the genetic components involved in *Brassica rapa* in the physiological and genetic mechanisms of gene-gene-gene re-

lationships between a plant and various races of the causative agent of vascular bacteriosis.

Techniques. We studied two mapping populations of doubled haploid lines (DHL) of *B. rapa*, the DH38 (♀P175 × ♂P143) and DH30 (♀P115 × ♂P143) that were obtained at Wageningen University (Netherlands), by the culture of microspores of a single F_1 plant in each crossing combination of three the main phenotypically distinct subspecies of the species: oil yellow sarson (original sample YS-143, k-FIL500, male parent DHL P143), Chinese leafy/stalked cabbage (PC-175, variety Nai Bai Cai, first mother parent DHL P175) and root turnips (VT-115, variety Kairyou Hakata, second maternal parent DHL P115) [15]. The lines of mapping populations DH30 and D38 were genotyped, respectively, with 299 and 294 AFLP and SSR markers. For the SSR analysis, we used 100 markers that were developed in the Multinational Brassica Genome Project (MBGP) (http://www.brassica.info).

For artificial infection, we used strains of four races of *Xanthomonas campestris* pv. *campestris* (Pam.) Dow. (hereafter *Xcc*) — PHW231 (race 1), HRI5212 (race 3), HRI1279a (race 4), B-32 (race 6) (strains were courtesy of Dr J. Vicente and Dr N.W. Schaad). The bacteria were stored at –84 °C; the inoculum (10^6 cells/ml) was obtained from a 2-day culture in the Kings B medium.

The evaluation of 64 duplicated haploid lines of mapping populations (26 population lines of DH30 and 38 population lines of DH38) and their parental forms for *Xcc* resistance took 2 years. The plants were grown in pots of 10 cm in diameter, in a greenhouse at 20/16 °C (day/night) and a 16-hour photoperiod until 3-4 true leaves. Three true leaves on each plant were inoculated in approximately 10 points, pinching half of the leaf near the veins 2-3 mm from its edge, with surgical forceps dipped in the bacterial suspension [16]. Each plant was inoculated with all *Xanthomonas* isolates. After inoculation, the plants were placed in a humid chamber for 24 hours, then in a greenhouse at 24 °C (day/night) for 2 weeks. The first signs of vascular bacteriosis appeared 10 days after inoculation. Accounting was carried out on a 4-point scale: 0 — no signs, 1 — necrosis around the point of inoculation (hypersensitivity reaction, HSR); 2 — necrosis around the inoculation point and chlorosis up to 0.5 cm in diameter; 3 — development of typical V-shaped necrosis. Annually, 3 independent tests were performed at different times, with 2 replications for each sample. Resistant samples were additionally inoculated to confirm the result.

To study the nature of the inheritance of the reaction to the pathogen in the winter greenhouse conditions, we compared 33 combinations of back-crossed plants with contrasting resistance to a particular race. The crossing was repeated 3 times, using forced pollination, with the application of pollen on castrated buds 3-4 days before the blooming. After the autogamic pollination of F_1, F_2 progeny were obtained, in which the splitting was evaluated by resistance to pathogen strains.

The significance of differences between the samples in the proportion of plants resistant to each race was determined by analyzing the variance, using the χ-square test at a statistical significant level of 95% [17]. The interdependence between the response of samples to different races of the pathogen was established by the Pearson correlation analysis [18], using Statistica 6.0 software (StatSoft, Inc., USA).

The QTL analysis of 64 doubled haploid lines of two mapping populations of *B. rapa* DH38 and DH30 was performed as described above [15], using MAPQTL 6.0 [19] software to establish the presence and location of candidate loci in the linkage group (5 cM mapping interval), LOD values (logarithm of odds) ($P = 0.05$) and degrees of variation of QTL-related resistance traits to dif-

ferent races, for each indicator of resistance and each population. The significance of each LOD was established by the permutation method (1000 repetitions). To establish the number and exact location of the identified QTLs on the genetic map, we used interval mapping assuming one QTL in the interval between the linked markers with a certain degree of recombination [12]. In order to efficiently identify linkage groups and establish their number, we used an integrated genetic map of *B. rapa* with a total length of 1068 cM, saturated with molecular AFLP and SSR markers [15]. AFLP and SSR markers, due to their neutrality [12, 14], cover the entire *B. rapa* genome, with the distance between markers of 2.27 cM on average. The genetic values of each of the possible genotypes in the two marker loci were expressed as a function of the values of the respective QTL and determined the frequency of recombination between the QTL and its flanking markers. When translating recombination data into distances in the linkage groups we used Kosambi map function [20].

To verify associations, a marker/trait was genotyped using MBGP's SSR markers, for which we found a link to resistance to certain *Xcc* races: BRMS-014, BRMS-043, BRMS-050, BRMS-051, BRMS-096 (Japan), SSR-87 and SSR-89 (China), Na10D09, Na12E02, Na12H09 and Ra2E12 (United Kingdom) (https://vegmarks.nivot.affrc.go.jp/VegMarks/app/page/marker). DNA for the PCR analysis was extrcted from young green leaves as described [21]. The PCR was performed in a 12.5 μl mixture containing 10× incubation buffer (1.25 μl), 0.25 μl dNTP mixture (10 mM), 0.25 μl each of the recommended primers (10 pmol/μl), 0.1 μl of Taq DNA polymerase (5 U/μl) (QBiogene, Germany) and 20 ng of genomic DNA. Amplification was carried out in a thermal cycler C-1000 (Bio-Rad, USA). PCR mode: 94°C 3 min; 30 cycles — 94°C 30 s, 55°C 30 s, 72°C 30 s; final elongation 75°C 7 min; then 4°C without time limit. Amplification products were separated by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and documented using the BioDoc system (Bio-Rad, USA).

Results. The studied lines (Table 1) demonstrated a reaction from resistance to high susceptibility. In general, similar results were obtained in different seasons, although a higher susceptibility of some plants was noted in the spring. Genotypes that exhibit immunity or hypersensitivity to all four races of the pathogen have been distinguished: in DH30 lines 35A, 69B, 98E; in DH38 lines 1g, 32d, 97a, 172a. In the DH30 population, responses to infection with strains B-32 (race 6) and 1279a (race 4) ($r = 0.64$), as well as HRI5212 (race 3) and PHW231 (race 1) ($r = 0.43$) were the most correlated. There was a significant correlation (r from 0.54 to 0.73) between the response to individual strains and the total damage of plants. Three strains turned out to be resistant to all strains of the pathogen. When inoculated with races 3, 4 and 6, the lines demonstrated susceptibility more often. The distribution of lines by response to infection with races 1 and 3 most closely corresponded to the monogenic inheritance of the trait. Segregation by the response to the strain B-32 of the most virulent race 6 (increase in the number of susceptible lines) was the most different from what was expected. In the DH38 population, there was a significant relationship between the response to strains B-32 (race 6) and 1279a (race 4) ($r = 0.52$), PHW231 (race 1) ($r = 0.4$), and between the response to individual strains and the general damage (r from 0.58 to 0.78). Four lines were resistant to all tested pathogen strains. The segregation for the response to PHW231 strain (increase in the number of susceptible lines in the DH38 population) deviated the most from what we expected. The distribution of lines by sensitivity to race 4 most closely corresponded to the monogenic inheritance of the trait (see Table 1).

1. Results of artificial infection of plants mapping populations of doubled haploid lines of *Brassica rapa* with *Xanthomonas campestris* pv. *campestris* (Pam.) Dow.

Line	Autumn and winter				Total points by year	Spring			
	B-32	1279a	5212	231		B-32	1279a	5212	231
DH30 (♀P115 × ♂P143)									
P1 115	0	1.0	2.0	0	3/4	0	2.0	2.0	0
P2 175	0	1.0	0	0	1/6	2.0	2.0	0	2.0
P3 143	0	0	2.0	0	2/2	0	0	2.0	0
6A	2.0	2.0	1.0	2.0	7/8	2.0	2.0	2.0	2.0
$18M_2$	1.0	0	0	0	1/4	2.0	1.0	1.0	0
28A	2.0	1.0	1.0	0	4/7	2.0	2.0	2.0	1.0
35A	1.0	0	0	0	1/4	1.0	1.0	1.0	1.0
38B	1.0	1.0	2.0	2.0	6/6	1.0	1.0	2.0	2.0
44A-V2	3.0	3.0	1.0	1.0	8/8	3.0	3.0	1.0	1.0
67	2.0	0	2.0	2.0	6/6	2.0	0	2.0	2.0
69B-1	1.0	1.0	1.0	0	3/3	1.0	1.0	1.0	0
79C-2	1.0	1.0	2.0	0	4/4	1.0	1.0	2.0	0
97	3.0	3.0	1.0	1.0	8/8	3.0	3.0	1.0	1.0
98E-2	1.0	1.0	1.0	1.0	4/4	1.0	1.0	1.0	1.0
110A-3	2.0	1.0	2.0	0	5/5	2.0	1.0	2.0	0
127c	1.0	1.0	3.0	3.0	8/10	2.0	2.0	3.0	3.0
160A	1.0	2.0	1.0	1.0	5/8	2.0	2.0	2.0	2.0
163A-2	2.0	2.0	1.0	2.0	7/7	2.0	2.0	1.0	2.0
164A-A^h	2.0	2.0	2.0	1.0	7/3	2.0	0	0	1.0
178A	2.0	1.0	0	1.0	4/7	2.0	2.0	1.0	2.0
189-A	2.0	1.0	3.0	3.0	9/10	2.0	2.0	3.0	3.0
206A^h	2.0	2.0	3.0	1.0	8/8	2.0	1.0	2.0	3.0
215c-1	3.0	2.0	2.0	2.0	9/9	3.0	2.0	2.0	2.0
238A	1.0	2.0	1.0	1.0	5/6	1.0	2.0	2.0	1.0
251-1	1.0	2.0	2.0	1.0	6/6	1.0	2.0	2.0	1.0
94	3.0	3.0	1.0	нд	7/8	3.0	3.0	2.0	0
113 i	3.0	3.0	2.0	нд	8/10	3.0	3.0	2.0	2.0
188A-3	1.0	2.0	1.0	нд	4/6	1.0	2.0	1.0	2.0
192H-1^h	3.0	3.0	3.0	нд	9/12	3.0	3.0	3.0	3.0
Resistant:susceptible	11:15	11:15	14:12	14:8		8:18	10:16	10:16	13:13
DH38 (♀P175 × ♂P143)									
1g-2	1.0	0	0	1.0	2/2	1.0	0	0	1.0
13a-VI	1.0	1.0	1.0	2.0	5/5	1.0	1.0	1.0	2.0
15в-3	0	0	2.0	1.0	3/3	0	0	2.0	1.0
23a-VI	2.0	1.0	2.0	2.0	7/7	2.0	1.0	2.0	2.0
25a-VI	2.0	1.0	1.0	2.0	6/6	2.0	1.0	1.0	2.0
32d-1	1.0	1.0	0	0	2/2	1.0	1.0	0	0
36c-3	3.0	3.0	2.0	2.0	10/10	3.0	3.0	2.0	2.0
40c-1^h	3.0	3.0	3.0	3.0	12/12	3.0	3.0	3.0	3.0
44a-1	3.0	2.0	2.0	0	7/7	3.0	2.0	2.0	0
51 f-1	1.0	2.0	2.0	2.0	7/7	1.0	2.0	2.0	2.0
52a-1	3.0	1.0	1.0	3.0	8/8	3.0	1.0	1.0	3.0
55h-1	2.0	1.0	2.0	1.0	6/6	2.0	1.0	2.0	1.0
57d-V3	0	0	1.0	2.0	3/3	0	0	1.0	2.0
58d-1	2.0	3.0	2.0	2.0	9/9	2.0	3.0	2.0	2.0
59a-2^h	2.0	3.0	1.0	2.0	8/6	2.0	0	2.0	2.0
62a-2^h	2.0	3.0	3.0	3.0	11/12	3.0	3.0	3.0	3.0
64-b-V-2^h	3.0	1.0	2.0	2.0	8/6	3.0	0	0	3.0
65a-2	3.0	2.0	1.0	2.0	8/8	3.0	2.0	1.0	2.0
72a-VI	2.0	2.0	1.0	1.0	6/6	2.0	2.0	1.0	1.0
75a-1	1.0	1.0	2.0	2.0	6/6	1.0	1.0	2.0	2.0
76в-1	1.0	2.0	1.0	1.0	5/8	3.0	3.0	1.0	1.0
78a-1	2.0	2.0	3.0	3.0	10/9	2.0	3.0	3.0	1.0
80a-1^h	2.0	2.0	2.0	2.0	8/12	3.0	3.0	3.0	3.0
92в-1	3.0	2.0	1.0	1.0	7/7	3.0	2.0	1.0	1.0
95a-1	3.0	1.0	3.0	2.0	9/9	3.0	1.0	3.0	2.0
97a-3	0	0	1.0	1.0	2/2	0	0	1.0	1.0
103a^h	3.0	3.0	0	2.0	8/7	3.0	0	1.0	3.0
123a	1.0	2.0	2.0	2.0	7/7	1.0	2.0	2.0	2.0
124a-1	1.0	3.0	2.0	2.0	8/8	1.0	3.0	2.0	2.0
127a-1	3.0	2.0	2.0	2.0	9/9	3.0	2.0	2.0	2.0
134a-4	3.0	1.0	2.0	2.0	8/8	3.0	1.0	2.0	2.0
136VI	0	3.0	3.0	3.0	9/8	1.0	1.0	3.0	3.0
142b-2	1.0	1.0	2.0	2.0	6/6	1.0	1.0	2.0	2.0
151a-2	1.0	1.0	1.0	2.0	5/5	1.0	1.0	1.0	2.0
154в	0	0	2.0	2.0	4/4	0	0	2.0	2.0

Table 1 continued

160c	0	0	2.0	1.0	3/3	0	0	2.0	1.0
169a-1[h]	2.0	1.0	2.0	2.0	7/6	2.0	0	2.0	2.0
172a	0	0	1.0	0	1/1	0	0	1.0	0
Resistant:susceptible	17:21	20:18	15:22	11:26		16:22	23:25	15:23	12:26

Note. B-32 — strain B-32, race 6; 1279a — strain HRI1279a, race 4; 5212 — strain HRI5212, race 3; 231 — strain PHW231, race 1. Sensitivity rating: 0 — no signs, 1 — hypersensitivity, 2 — weak susceptibility (affected area less than 5 mm or chlorosis), 3 — susceptibility, development of typical symptoms; [h] — lines used for hybridization.

2. Segregation in F_2 after crossing plants with contrasting resistance, selected in the mapping populations of the doubled haploid lines of *Brassica rapa* in artificial infection with *Xanthomonas campestris* pv. *campestris* (Pam.) Dow.

Crossing combination	Number of plants	B-32	1279a	5212	231
DH30 (♀P115 × ♂P143)					
206(r) × 192(s)	56	0:56	42:14[a, *]	0:56	0:56
192(s) × 206(r)[r]	64	0:64	50:14[a, *]	49:15[a, *]	0:64
164(r) × 192(s)	48	0:48	32:16[a]	37:11[a, *]	0:48
192(s) × 164(r)	36	0:36	26:10[a, *]	24:12	0:36
DH38 (♀P175 × ♂P143)					
80(s) × 64(r)	36	0:36	28:8[a, *]	26:10[a, *]	3:33
64(r) × 80(s)	42	1:41	31:11[a, *]	28:14[a]	1:41
62(s) × 59(r)[r]	40	32:8*	26:14[a]	0:40	0:40
59(r) × 62(s)	28	0:28	0:28	0:28	0:28
40(s) × 64(r)	48	0:48	0:48	30:18[a]	0:48
64(r) × 40(s)	64	0:64	49:15[a, *]	50:14[a, *]	0:64
62(s) × 136(r)	20	0:20	0:20	0:20	0:20
136(r) × 62(s)	20	0:20	16:4[a, *]	0:20	0:20
80(s) × 169(r)[r]	24	0:24	20:4[a]	0:24	0:24
169(r) × 80(s)	20	0:20	0:20	0:20	0:20
62(s) × 64(r)	56	0:56	0:56	0:56	0:56
64(r) × 62(s)[r]	60	0:60	0:60	45:15[a]	0:60
62(s) × 103(r)	24	0:24	18:6[a]	0:24	0:24
103(r) × 62(s)	35	0:35	28:7[a, *]	1:34	2:33
169(r) × 62(s)[r]	27	0:27	18:9[a]	0:27	0:27
62(s) × 169(r)	18	0:18	0:18	0:18	0:18
78(s) × 59(r)	28	0:28	0:28	0:28	20:8[a, *]
59(r) × 78(s)[r]	49	0:49	40:9[a]	0:49	35:14[a]
78(s) × 103(r)	56	0:56	40:16[a]	0:56	43:13[a, *]
103(r) × 78(s)	68	0:68	50:18[a, *]	0:68	48:20[a]

Note. B-32 — strain B-32, race 6; 1279a —strain HRI1279a, race 4; 5212 —strain HRI5212, race 3; 231 —strain PHW231, race 1; r — resistant parental line, s — susceptible parental line, [r] — a combination with a reciprocal effect on plant response to infection with a pathogen; [a] — combinations with segregation close to 3:1.
* Segregation corresponding to the expected 3:1 is reliable at 95% accuracy according to the χ-square test.

When studying the nature of the inheritance of the plant sensitivity to the pathogen, we selected for hybridization 20 lines, i.e. 7 susceptible to all strains of the pathogen, 13 resistant to only one race and susceptible to the rest (Table 2). Crossing was easy, resulting in 15-20 seeds per pods. The lines with a reduced self-compatibility, which set 6-7 seeds per pod with autogamous pollination, were rejected. All F_1 plants of both mapping populations showed resistance to at least one out of 4 races of the pathogen. After autogamous pollination, F_1 plants yielded 20 populations of F_2 from the mapping DH38 population lines and 4 F_2 progeny from the DH30 population lines, in which we identified segregation by resistance to the four pathogen strains. The observed ratio of the resistant and susceptible forms in F_2 and the subsequent analysis of hybrids of the selected resistant and susceptible lines showed that the race-specific resistance is mainly determined by a single dominant locus (see Table 2).

Three out of four populations of F_2 DH30 showed monogenic resistance to race 4 (strain HRI1279a) and to race 3 (strain HRI5212), and one showed resistance with a possible deviation from the monogenic (shift toward susceptibility). Interestingly, the parent lines 206 and 164 (see Table 1) were resistant only to race 1. Among the twenty F_2 populations from the crossing of lines of the mapping population DH38, ten had monogenic resistance to race 4 (strain HRI1279a), including resistance with deviation from 3:1 segregation; five had monogenic resistance to race 3 (strain HRI5212); four had monogenic resistance to race 1 (strain PHW231); and one had monogenic resistance to race 6 (strain B-32). Obviously, the resistance to race 4, which is dominant in populations of *B. rapa* [7] species, retained its significance when crossing parental lines (even if the parent has a susceptibility reaction). In 8 out of 12 pairs of parents, the reciprocal effect of crossing was manifested, which gives a possible explanation for

the appearance of stability in the progeny of the two original parental lines that are susceptible to race (when creating F_1). Of these eight pairs, six had reciprocal asymmetry of reaction to infection by race 4; three had reciprocal asymmetry of response to infection with race 3; one pair showed reciprocal asymmetry to race 6; asymmetry to race 1 was not observed.

3. Results of QTL analysis of resistance to *Xanthomonas campestris* pv. *campestris* (Pam.) Dow. in mapping populations of doubled haploid lines *Brassica rapa* in artificial infection

Strain (race)	Linkeage groups	LOD score (minimum-maximum)	Percentage variation of the trait phenotypic variability, % Expl.
	DH30 (♀P115 × ♂P143)		
PHW231 (race 1)	A03, A07	1.09-1.14	19.7-21.1
HRI5212 (race 3)	A01, A03, A06, A07	1.16-2.35	19.9-36.3
HRI1279a (race 4)	A02, A03, A05	0.77-3.08	13.7-44.6
B-32 (race 6)	A01, A03, A04, A05, A07, A09	1.02-1.82	17.8-29.5
	DH38 (♀P175 × ♂P143)		
PHW231 (race 1)	A02, A04, A06, A08	0.75-2.31	9.7-26.9
HRI5212 (race 3)	A01, A05, A10	0.72-1.81	9.3-21.8
HRI1279a (race 4)	A03, A06	1.06-2.93	13.4-32.7
B-32 (race 6)	A01, A03, A04, A06, A08	0.66-3.44	8.5-37.2

QTL analysis (Table 3, Fig. 1) (Fig. 1, see the online version of the article on the website http://www.agrobiology.ru) revealed 13 QTLs controlling resistance to four races of vascular bacteriosis pathogen in DH30. At the same time, 10 loci stably maintained localization in both years of the research (LOD 0.77-3.80; loci with LOD > 1.1 are usually considered). These are loci at the top of linkage groups A01 and A03 which control resistance to race 3 of the pathogen, 2 loci at the bottom of A03, controlling resistance to races 3, 4 and 6, loci in the middle of A05 (they control resistance to race 4, also to race 6 in spring), and 2 QTLs in the lower part of A07 for resistance to race 3, also to race 6 in the autumn and winter and to race 1 in spring). QTL associated with resistance to race 6 was found in the middle of the linkage groups A01 and A04 and in the lower half of A09 (the position of QTL in A09 differed by 7 cM among years). The variability explainable by the found QTLs ranged from 13.7 to 44.6%. The effects of genes in all established loci are additive. The highest and most stable LOD values (in terms of years) were characteristic of three QTLs located in the upper part of A01 and at the bottoms of A03 and A07. Therefore, the most important loci responsible for the manifestation of resistance to different races of the causative agent of vascular bacteriosis in DH30 *B. rapa* are in the linkage groups A01, A03 and A07.

For the DH38 population, 19 QTLs were revealed (see Table 3), and all loci consistently maintained localization during study (LOD 0.66-3.44). Six QTLs controlled resistance to races 1 and 4, 4 — to race 3, and 8 — to race 6. At the same time, four loci of resistance to races 4 and 6 were located in the middle and lower parts of A03, three loci of resistance to races 1, 4, and 6 were located in the middle and lower parts of A06, and three loci of resistance to races 1 and 6 were located in the upper part of A08. That is, the most significant loci of resistance to vascular bacteriosis agent in the DH38 lines are located in linkage groups A03, A06 and A08. At the top of A10, the earlier mapped CAPS marker of the locus of resistance to race 5212-I *FLC1* was found, which is also associated with many important physiological and biochemical characteristics (transition time to the generative phase, ascorbic acid and carotene content) [15].

The QTLs that control resistance to races 3 and 6 in the lines of both mapping populations were located in close positions in A01; to races 4 and 6 — at the bottom of A01, and to race 6 — in the middle of A04. For both mapping populations, the A03 loci were the most important for the genetic control of re-

sistance to *Xcc*. Note that at the bottom of the A03 group we found the previously mapped locus *BrFLC5* which controls the most important features: transition time to flowering, productivity and biochemical composition [15].

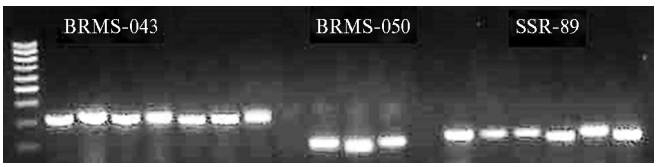

Fig. 2. An example of PCR analysis of the susceptible (+) and resistant (–) to *Xanthomonas campestris* pv. *campestris* (Pam.) Dow. lines of mapping populations DH30 (♀P115 × ♂P143) and DH38 (♀P175 × ♂P143) of *Brassica rapa* with markers BRM-S043, BRMS-050 and SSR-89: 1 — DH30/18 (–), 2 — DH30/97 (+), 3 — DH30/35 (–), 4 — DH38/1 (–), 5 — DH38/36 (+), 6 — DH38/62 (+), 7 — DH38/160 (–); 9 — DH30/17 (+), 10 — DH30/178 (–), 11 — DH30/192 (+); 13 — DH30/35 (–), 14 — DH30/192 (+), 15 — DH30/238 (–), 16 — DH30/18 (–), 17 — DH30/160 (–), 18 — DH30/206 (+);the leftmost lane is a 100 bp molecular weight marker (10 fragments from 100 to 1000 bp) (SibEnzyme, Russia). The expected amplicon size for BRMS-043 is 318 bp, for BRMS-050 164 bp, for SSR-89 199 bp.

SSR analysis of lines of both mapping populations contrasting in resistance (Fig. 2) showed that the molecular marker BRMS-043 associated with resistance to race 4 in the DH30 population and to three races in the DH38 population is present in the expected position (318 bp) in resistant DH38 lines and in a position close to the expected in stable DH30 lines (216 bp). The amplicon size is 321 bp in the susceptible line 97 of the DH30 population, and 312 bp in the susceptible lines 36 and 62 of the DH38 population. The BRMS-050 marker associated with resistance to race 3 in DH30 population is present in the expected position (164 bp) in the stable line 178, while in the susceptible lines 127 and 192 the length of the identified fragment is 180 bp. The SSR-89 marker associated with the locus of resistance to race 3 and (with a minor LOD) to race 6 in the DH30 population is detected in the expected position (199 bp) in the resistant lines 18 and 35. The susceptible lines 192 and 206 (damage score 3), as well as the line 238 (previously assessed as stable) have a 204 bp fragment, and the previously stable line 160 has a 210 bp fragment. However, it should be noted that resistant lines 18 and 35 show no signs of damage, and the lines with controversial results show a hypersensitivity reaction. The BRMS-051 marker of the locus of resistance to race 5212 in the DH30 population is in the expected position of 262 bp in the stable line 18 and 5 bp above in the stable line 69 with a hypersensitivity reaction; in the susceptible line 206, the fragment size is 150 bp. The susceptible line 44 of the DH38 population carries Na12E02 marker associated with the locus of resistance to races 4 and 6 (the expected fragment length is 132 bp). Stable line 160 and line 52 with a hypersensitivity to race 4 and susceptibility to race 6 do not have this marker.

In summary, we found microsatellite markers of *B. rapa* loci of resistance to several races of the causative agent of vascular bacteriosis. In most cases, resistant genotypes with no signs of damage carry the marker in the expected position. It should be noted that the influence of the maternal cytoplasm on the manifestation of a stable phenotype, which we first identified in *B. rapa* in this work, most likely can significantly affect the localization and effect of the identified chromosomal QTLs. However, there is no recognized method for taking into account the interaction of nuclear and cytoplasmic genes in mapping plant resistance.

Vascular bacteriosis is especially harmful for cabbage *Brassica oleracea* L. [1], although it affects almost all species of the *Brassica* genus, including weed cruciferous and ornamental plants, and leads to significant annual economic losses. However, little is known about the genetic components and physiological

mechanisms of resistance to this disease in plants. Since phytopathogenic bacteria interact with plants according to the gene-to-gene principle, they are a convenient object for molecular genetic studies of relationships in the pathogen—plant system. In practice, knowledge of such mechanisms is important for the creation of resistant varieties which is the most economical and environmentally friendly method to combat the disease.

Obtaining resistant genotypes is complicated by the existence of at least nine races of the pathogen. The widespread races 1 and 4 are the most dangerous for the species *B. oleracea* [22, 23], therefore resistance to these two races is the minimum necessary condition for controlling the spread of vascular bacteriosis in cabbage plants. In studies of the *B. oleracea* samples, Dr J.D. Taylor and the team [3] found that resistance to races 1 and 4 was very low or absent, while resistance to less common races 2, 3 and 6 was relatively common. In contrast, race-specific resistance to races 1 and 4 is widely represented in other species of the genus *Brassica*, i.e. in *B. rapa*, *B. carinata* [3, 9], *B. napus* and *B. nigra* [24-26]. However, genetic control and mechanisms of resistance to different races of *Xanthomonas* in these species are still poorly understood. Moreover, not a single study has been conducted to identify and establish the chromosomal localization of quantitative trait loci that determine resistance to individual *Xcc* races in such an economically valuable species as *B. rapa*, the object of our research.

Bacteria are known to use effector proteins delivered to a plant cell by a Type 3 Secretion System (T3SS) to suppress constitutional stability stimulated by the microbe-associated metabolite profile (MAMPs). The variability of the presence, expression and amino acid sequence of the T3SS effectors depends on the race of the pathogen and reflects its host plant specificity [2]. The corresponding plant resistance genes respond to such inhibition of expression of resistance. Bacterial genes responsible for interaction with the plant are able to influence the general physiological and genetic processes in bacteria. Unfortunately, the mechanisms of interaction between the physiological and genetic systems of plants and phytopathogenic bacteria have not yet been largely revealed. This is due, primarily, to the lack of information about the natural polymorphism in the population of phytobacteria and a simple model system for analyzing such interaction, which obstructs detection of the chromosomal localization of genes and/or loci that determine quantitative resistance to diseases.

In *B. napus*, PI199947, later identified as *B. carinata* [23, 27], the dominant race-specific resistance to the pathogen of vascular bacteriosis was described, which is widespread among plants with the B genome [3, 6]. Race-specific resistance is usually associated with a hypersensitivity reaction (HSR) at the point of entry of an incompatible *Xcc* race through hydatods, but often only a partial HSR is observed [23]. Identification of QTL using RFLP and RAPD markers has shown that the plant response is associated with several additive loci in different linkage groups [8]. Earlier, Russian scientists [9] found that the resistance gene to race 4 in Chinese cabbage (*B. rapa*) is about 3 cM from the cruciferous keel resistance locus. The resistance gene *Rxc4* in *B. napus* has also been mapped [10].

In the genome-wide sequencing of the *B. oleracea* genome, three minor and one main QTLs were detected which are determining in the progeny resistance to one race *Xcc* [28]. In the forms of parental inbred lines, the authors identified 674521 SNP, without, however, indicating neither the inbreeding generation, nor the number of individual plants from each of the parental forms whose DNA were isolated to establish an SNP. It did not take into account that all individuals of the family obtained from one F_2 individual were genetically identical to each other after *n* generations (with the exception of the remaining

heterozygous in generation n –1), and that the DNA for full genome sequencing was not isolated from the same plant. Obviously, because of this, the share of dCAPS markers created by the authors and suitable for analysis was only 70%, and only half of them could be used to saturate the existing *B. oleracea* basemap. In addition, to create a genetic map and QTL mapping, Korean researchers [27] used the F_2 population with an average interval between markers of 3.88 cM, which is 1.61 cM higher than the average distance between the markers of the mapping populations of *B. rapa* haploid doubled lines in our studies. Since the Korean team used the cumulative DNA of the F_3 plants for mapping, without indicating that the F_3 plants were obtained as a result of individual self-pollination in F_2, in this case the expected segregation for one locus is not 1:2:1, but 3:2:3 because a heterozygous locus in F_2 has only one chance out of two to be fixed in F_3. The lines of doubled haploids are completely devoid of these shortcomings and can also be attributed to the so-called "immortal" populations, since they do not need to be re-created and each time saturated with molecular markers during QTL mapping, especially if it is carried out in different years and/or different ecological and geographical areas [12]. It should also be noted that we are not aware of any work in which SNP stability issues would be considered taking into account the frequency of both natural and artificial mutagenesis, recombinogenesis (primarily at the level of a single nucleotide) and the degree of degeneracy of the genetic code in the coding and non-coding regions of the genome. In our opinion, this would allow for more precisely limits of applicability of this type of molecular markers. Finally, the mapping of chromosome loci which determine the specific resistance of plants to each of the four races of *Xcc* has never been described until this work.

We found chromosomal loci of resistance to races 1 and 4 of the pathogen in the linkage group A06 of the mapping population DH38 lines and additionally to race 4 in the A02 group of DH30, which confirms the results of the QTL mapping of resistance to races 1 and 4 of *X. campestris* in the F_2 population from crossing high-inbred lines of yellow sarson R-o-18 and self-pollinated sample of Chinese cabbage B162 [11]. To date, this is the only study known to us which identifies the exact number and location of the QTLs that determine the resistance to two *Xanthomonas* races. In our studies, QTL resistance to four *Xanthomonas* races has been identified and mapped, for three of which QTL mapping has not previously been carried out. In addition, we established the effect of each QTL and the percentage of phenotypic variability caused by each of the identified QTLs for each race of the pathogen. Hybridological analysis established predominantly monogenic inheritance of the resistance. We also revealed the block structure of individual parts of the genome and the block character of inheritance. It has been shown that a single genomic block (QTL) may be responsible for resistance to different races of the pathogen. Molecular markers genetically linked to localized QTLs have been identified. DHL screening of *B. rapa* was carried out, and the species effective molecular genetic descriptors for resistance to vascular bacteriosis (for each race separately and for the pathogen in general) were identified.

In summary, we have for the first time identified and localized linkage groups and chromosome loci involved in the formation of the physiological stability of *Brassica rapa* to four races of *Xanthomonas campestris* pv. *campestris*. The lines of doubled haploids that are resistant to all the studied races of the pathogen of vascular bacteriosis are revealed. These lines can be included in the scientific and breeding programs as sources of the resistance. The obtained data is important for understanding the mechanism of *B. rapa* race specific resistance and, in the future, it can serve as a basis for an accurate quantitative assessment

of the effects of avr/pth genes and identified QTLs, as well as improvement of the methodology of similar work with other traits.

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APPLICATION OF SSR MARKERS FOR STUDY OF GENETIC DIVERSITY OF *Venturia inaequalis* IN THE DIFFERENT TYPES OF ORCHARDS IN THE NORTH CAUCASIAN REGION

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Abstract

Apple scab caused by ascomycete fungus *Venturia inaequalis* (Cooke) G. Winter is one of the most harmful diseases of apple trees, which leads to significant economic losses in apple production in the world. North Caucasus is a region with climatic conditions favorable for *V. inaequalis*. Therefore, the creation of resistant varieties is an important target for apple breeding. Study of the genetic diversity of the pathogen is an integral part of both science-based apple breeding programs and systems of protection against the pathogen. This paper is the first report on SSR analysis of genetic diversity of *V. inaequalis* strains collected in apple orchards that differ in structure and are located geographically remotely in the Krasnodar Territory and the Republic of Adygea. To study the genetic polymorphism of the phytopathogen populations, two industrial gardens and a collection of *Malus orientalis* were surveyed in the Kuban and Caucasus foothill agro-ecological zones of the region. The genetic heterogeneity of the host plant populations at the sampling sites varied significantly, since the industrial orchards were single-cultivar plantations of the apple varieties Gala, Renet Simirenko, Golden Delicious, and Champion while in the collection garden the accessions originated from different parts of the *M. orientalis* natural area. Eight SSR markers used were 1aac4f, Viga7/116, Vitc1/2, Vitcca7/P, Vicacg8/42, Viga3/z, 1tcla, Vitc2/D. The number of alleles per locus revealed in SSR analysis of 36 monosporic isolates of *V. inaequalis* was 4 for 1aac4f, 6 for Vitc2/D, 10 for Viga7/116 and Vicacg8/42, 11 for Vitcca7/P, and 12 for Vitc1/2 and 1tcla. Upon the whole, there were 4 (1aac4f) to 12 alleles (Vitc1/2, 1tcla) for polymorphic markers, and only one allele was detected for marker Viga3/z. Despite the fact that some markers showed various distributions of identified alleles in all subpopulations, these differences were not sufficient to differentiate the subpopulations. UPGMA-analysis showed no relationship between clusterization and the geographical origin of the isolates, indicating low inter-population differences. This can indicate a free gene flow between the populations due to human activity as they are too distant from each other to allow natural transfer of spores. The obtained results suggest significant genetic diversity in the investigated set of monospore isolates. Genetic diversity was higher in the *V. inaequalis* population from the *M. orientalis* collection, indicating the effect of plant population heterogeneity on genetic polymorphism of the pathogen. In our opinion, the differences in polymorphism for some SSR markers, when compared our data and the results reported by other researchers' for European populations of *V. inaequalis*, could be due to genetic differences in populations of *V. inaequalis* from North Caucasus region and the European populations.

Keywords*:* apple scab, *Venturia inaequalis*, genetic diversity, SSR-markers, allele polymorphism, North Caucasus

Apple scab caused by ascomycete *Venturia inaequalis* (Cooke) G. Winter is one of the most harmful diseases of apple trees, which leads to significant

economic losses in apple production in the world [1]. During epiphytotics the scab may affect up to 80-100% of apple production sensitive thereto in areas with conditions favoring scab growth (basically, temperate countries including Russia, and North Caucasus region, in particular) [2]. High quality requirements to the production demand strict control over the disease. As a rule, fungicides are used for this purpose nowadays, with a number of treatments being 17-22 applied to highly-susceptible varieties in season [2].

Great attention is paid to development of resistant cultivars due to high harmfulness of scab. Analysis of genetic diversity of pathogens is important both for making breeding programs and disease control measures against pathogens. Application of artificial infection background for choosing resistant cultivars from hybrid materials will enable to intensify the breeding process. Therewith, the information on genetic diversity of pathogen will enable us to make the most heterogeneous inoculum, thereby increasing efficiency for assessing stability [3].

Morphological and cultural characteristics and virulence determinants [4-9] were used in early steps for analyzing genetic diversity of pathogen. However, application of DNA markers that opened a new phase in studying genetic diversity of *V. inaequalis* [10] proved to be the most efficient. In early 1990s marker analysis of *V. inaequalis* in major cases was based on three methods, i.e. RFLP (restriction fragment length polymorphism), RAPD (random amplification of polymorphic DNA) and ITS sequence analysis [10, 11]. More informative DNS-markers like SSR (simple sequence repeats) have appeared along with the development of molecular-genetic tools. They are characterized by high specificity and sensitivity, relative simplicity in operation and estimated results [10]. A number of genetic diversities of pathogen was done with their application. According to major experiment made by some scientists from France, Belgium and China *V. inaequalis* was found in Central Asia in the center of *Malus* spp. origin [12, 13]. The genetic diversities of *V. inaequalis* populations found in *Malus sieversii* in Central Asia were presented by more types than in European populations in *Malus* × *domestica* and *Malus sylvestris* [13] and indicated age of their existence. Dependence of genetic diversity of *V. inaequalis* on population age was also noted by other authors [14, 15]. High level of intrapopulation diversity and low differentiation between populations was demonstrated in many population analyses of this fungal pathogen [10, 11, 16, 17). Availability of vast panmictic populations of the pathogen at quite moderate potential of natural propagation (15-60 m) [18] is explained by high gene flow due to human activity (movement of affected seeds and fruit) [10, 13].

Expansion of geographical boundaries where genetic polymorphism is analyzed is likely to specify peculiarities characterizing both inter- and intrapopulation genetic interactions in *V. inaequalis*. Besides, analysis of genetic diversities of pathogen in both natural and man-made ecosystems of North Caucasus regions as a part of *M. orientalis* species will enable to evaluate microevolutionary processes of interactions between pathogen and host-plant. Up to now, such analyses were not conducted in the territory of Russia.

By using SSR markers we were the first in making SSR genotyping in geographically remote populations of *V. inaequalis* in North Caucasus and revealed polymorphism by a number of markers that were used at analysis of genetic structure of populations in this pathogen in other regions. Comparison between results obtained and information from literature enables us to assume that North Caucasus population of this pathogen may differ genetically from the European one.

The work includes analysis of diverse *Venturia inaequalis* isolates selected from agrophytocenoses different both by structure and localization by using SSR

markers.

Techniques. *Venturia inaequalis* sets (April-May 2015) were taken in three geographical points of two agro-ecological zones of North Caucasus regions, i.e. Prikubanskaya (No.1, Krasnodar, Vodniki Settl., ZAO Experimental Production Farm Tsentralnoe, 2nd division) and Predgornaya in the Republic of Adygea (No.2, Maikop region, Podgorny Settl., Maikop experimental station at All-Union Research Institute of Plant Breeding, collection of apple genetic resources; No.3, Abadzekhskaya village, Muskat farm household). Monospore isolates of agent were isolated from ascospores to pure culture in sterile conditions according to original procedure [19] using leaf litter with pseudothecia as per indicated protocols [20]. Agarized media were prepared by standard microbiological methods [21, 22]. Isolates in points 2 and 3 were taken from various trees of one species or cultivars (*M. orientalis* and Champion accordingly), and from various apple varieties (Gala, Renet Simirenko and Golden Delicious in point 1).

DNA was extracted as per recommendations [23].

Microsatellite DNA markers 1aac4f [10] and Viga7/116, Vitc1/2, Vitcca7/P, Vicacg8/42, Viga3/z, 1tcla, Vitc2/D [24] were used to assess genetic diversities. Mixture for PCR amplification (25 μl) contained 50-70 μg of DNA, 0.05 mM dNTPs, 0.3 μM of each primer; 2.5 μl of 10× reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA-polymerase. PCR (a Mastercycler gradient amplifier, Eppendorf, Germany) was performed by the following scheme: 5 min at 95 °C (initial denaturation); 10 s at 95 °C (denaturation), 30 s at 60 °C (primer annealing), 30 s at 72 °C (elongation) (30 cycles); 3 min at 72 °C. Size of amplified parts of SSR markers was identified (an automatic genetic analyzer ABI prism 3130, Applied Biosystems, USA). Data were processed with GeneMapper 4.1 application included to ABI prism 3130 software.

Cluster analysis was made by UPGMA method applying PAST software [25]. Allele frequencies were calculated with GenAlEx 6.5 [26], and PIC value (Polymorphism Information Content) with Polymorphism Information Content Calculator (http://w3.georgikon.hu/pic/english/kezi.aspx) [27].

Results. A total of 36 monospore isolates were used for the analysis (in points 1, 2 and 3 — 20, 9 and 7, respectively). Seven of eight SSR markers in the analyzed set at SSR genotyping proved to be polymorphous, i.e. from 4 to 12 alleles per locus were detected in them (Table 1). Marker Viga3/z with one allele (99 bp) was excluded from statistical processing as a monomorphic one.

1. Polymorphism of SSR markers in a set of *Venturia inaequalis* isolates from various agro-ecological zones (n = 36, North Caucasus region, 2015)

Parameter	SSR marker						
	1aac4f	Viga7/116	Vitc1/2	Vitcca7/P	Vicacg8/42	1tcla	Vitc2/D
Number of alleles per locus	4	10	12	11	10	12	6
Range of part lengths, bp	107-120	138-180	181-220	168-215	203-230	116-167	213-246
PIC	0.153	0.733	0.733	0.870	0.655	0.854	0.710

N o t e. PIC — polymorphism information content.

The least allele polymorphism was detected for markers 1aac4f and Vitc2/D. A number of both concordances and reverse results are worth noting for some regions by comparing data on SSR polymorphism level obtained by us and by foreign authors. So, in Worchestershire and East Malling of Great Britain when using four SSR markers for comparing genetic diversity and structure of *V. inaequalis* population the highest polymorphism was detected by SSR marker Vitc2/D (29 alleles per locus) by selecting among 102 monospore isolates, while the value for Vitcca7/P and Vitc1/2 markers was far lower, 19 and 9 alleles per locus, accordingly [14]. In our analysis Vitcca7/P and Vitc1/2 markers may be attributed to the most polymorphous. At the same time polymorphism values of

SSR markers 1aac4f and 1tcla detected both by I. Tenzer et al. [10] and by us on the contrary correspond to each other. These scientists have assessed genetic diversity in 11 *V. inaequalis* populations from five European countries (France, Germany, Italy, the Netherlands and Switzerland). According to them 1tcla marker, as was also indicated by us, possessed one of the highest values of allelic polymorphism (26 alleles per locus), therewith the value was one of the lowest by 1aac4f marker (4 alleles per locus). A group of scientists having made 21 SSR markers for *V. inaequalis* and assessed polymorphism thereof using 44 monospore isolates from six European countries have identified 8, 18, 9 and 11 alleles per locus for Viga7/116, Vitc1/2, Vitcca7/P and Vicacg8/42, respectively, at average value of 9 alleles per locus for 21 markers [24]. High polymorphism by these markers was also indicated in our paper. At the same time the highest number of alleles (24 per locus) [24] was reported for Vitc2/D marker, while in populations analyzed by us it proved to be one of the least polymorphous (6 alleles per locus).

In our opinion, great polymorphism differences by some SSR markers detected by us in North Caucasus subpopulations of *V. inaequalis* from that of described by foreign scientists for European population of this pathogen may indicate to genetic differences between North Caucasus and European population of *V. inaequalis* due to their geographical distance.

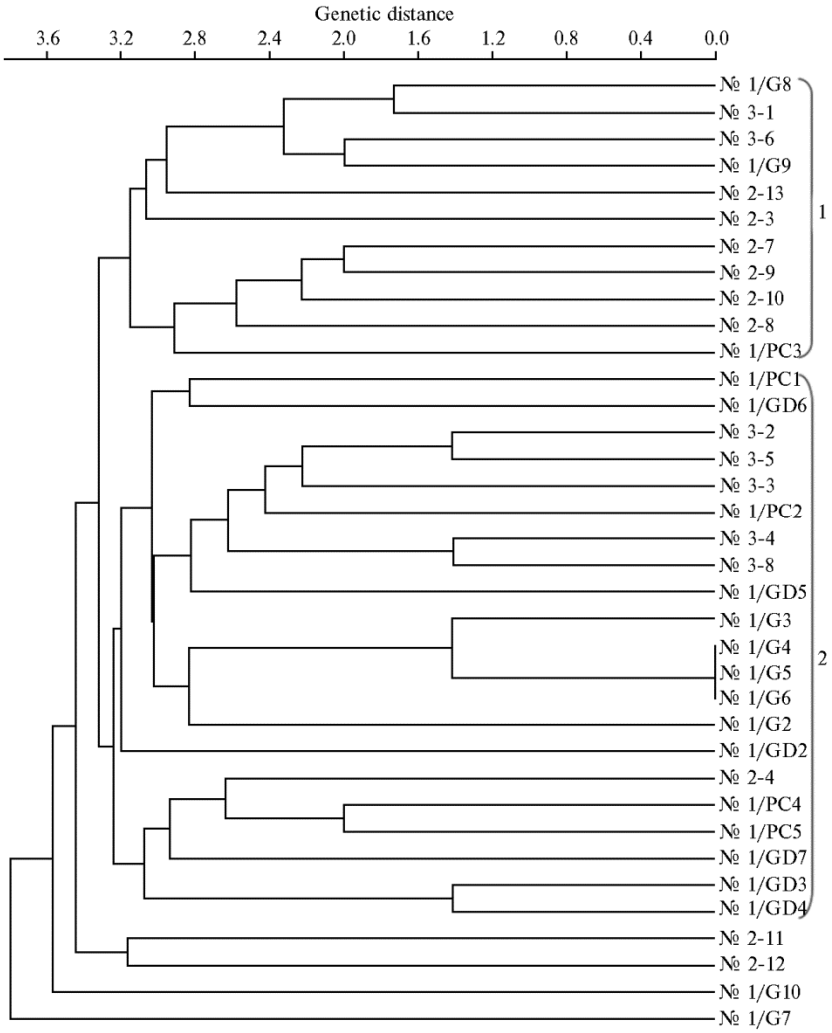
Comparison of allele frequency of SSR markers in set of monoisolates is given in Table 2 for assessing differences between three subpopulations.

2. Allele frequency detected by SSR genotyping of *Venturia inaequalis* isolates in three populations of pathogen from various agro-ecological zones (North Caucasus region, 2015)

SSR marker	No. 1 (n = 20)	No. 2 (n = 9)	No. 3 (n = 7)
1aac4f	107 (0.950); 120 (0.050)	107 (0.778); 109 (0.111); 112 (0.111)	107 (1.000)
Viga7/116	143 (0.025); 164 (0.175); 166 (0.050); 168 (0.350); 170 (0.125); 172 (0.075); 174 (0.125); 180 (0.075)	138 (0.111); 141 (0.111); 164 (0.444); 168 (0.333)	143 (0.143); 164 (0.286); 168 (0.571)
Vitc1/2	183 (0.175); 187 (0.025); 190 (0.100); 192 (0.200); 193 (0.050); 195 (0.300); 213 (0.050); 215 (0.100)	181 (0.111); 190 (0.222); 192 (0.222); 210 (0.333); 220 (0.111)	190 (0.286); 192 (0.286); 194 (0.429)
Vitcca7/P	0 (0.050); 168 (0.125); 194 (0.175); 196 (0.050); 200 (0.075); 202 (0.250); 204 (0.050); 210 (0.125); 215 (0.100)	170 (0.111); 196 (0.222); 198 (0.222); 200 (0.111); 202 (0.111); 204 (0.111); 215 (0.111)	194 (0.286); 198 (0.429); 200 (0.143); 202 (0.143)
Vicacg8/42	205 (0.025); 210 (0.575); 212 (0.050); 216 (0.100); 218 (0.050); 222 (0.100); 224 (0.050); 228 (0.050)	203 (0.222); 205 (0.333); 210 (0.222); 216 (0.111); 230 (0.111)	210 (0.857); 216 (0.143)
1tcla	116 (0.050); 120 (0.175); 129 (0.050); 131 (0.200); 135 (0.150); 141 (0.375)	133 (0.222); 137 (0.111); 139 (0.111); 143 (0.333); 145 (0.111); 167 (0.111)	120 (0.429); 133 (0.286); 135 (0.286)
Vitc2/D	213 (0.200); 232 (0.200); 234 (0.400); 236 (0.150); 244 (0.050)	232 (0.667); 234 (0.111); 236 (0.111); 246 (0.111)	234 (0.286); 236 (0.714)

N o t e. No. 1 — Krasnodar, Vodniki Settl., ZAO Experimental Production Farm Tsentralnoe, 2nd division (Prikubanskaya agro-ecological zone); No. 2 — Maikop region, Podgorny Settl., Maikop experimental station at All-Union Research Institute of Plant Breeding, collection of apple genetic resources, No. 3 — Abadzekhskaya village, Muskat farm household 3 (the Republic of Adygea, Predgornaya agro-ecological zone). Allele size is given in bp, allele frequency is indicated in brackets.

Majority of alleles detected in subpopulation 1 from Prikubanskaya agro-climatic zone is due to small sample size, i.e. 20 monospore isolates. It should be mentioned availability of alleles with maximum value of this indicator simultaneously in two or three subpopulations (allele 107 bp by marker 1aac4f, allele 168 bp by markerViga7/116 and allele 210 bp by marker Vicacg8/42). This fact may testify to small interpopulation differences in spite of



UPGMA-dendrogram characterizing high level of genetic similarities between monospore isolates of *Venturia inaequalis* from different agro-ecological zones: No. 1 — Krasnodar, Vodniki Settl., ZAO Experimental Production Farm Tsentralnoe, 2nd division (Prikubanskaya agro-ecological zone); No. 2 — Maikop region, Podgorny Settl., Maikop experimental station at All-Union Research Institute of Plant Breeding, collection of apple genetic resources, No. 3, Abadzekhskaya village, Muskat farm household 3 (the Republic of Adygea, Predgornaya agro-ecological zone) (North Caucasus region, 2015).

the presence of unique alleles in each sampling that in majority cases appeared occasionally. A group of monospore isolates from collection of *Malus orientalis* sets in Maikop experimental station at All-Union Research Institute of Plant Breeding is of interest. For some SSR markers (1aac4f, 1tcla and Vitc2/D), a number of alleles in this group consisting of 9 isolates has matched or exceeded the same in population No. 1 presented by 20 isolates. It may be considered as higher genetic diversity of *V. inaequalis* population to be found on *M. orientalis* plants from a collection of genetic resources well preserving a wide spectrum of specific sets of *Malus* species, as well as of apple tree varieties (point No.2). It should be mentioned that our results agree with data by O.N. Barsukova [5], who has compared diversity of pathogen in this collection and in the region under morpho-cultural and virulent characteristics. The author concludes that the diversity of pathogen found in wildlife host species in natural growing conditions and in collection planting is higher than in populations having formed

in cultivated species [5].

We have made a cluster analysis for assessing level of genetic similarity in monospore isolates from sampling to be studied (Fig.). Under clusterization results two main clusters and four sets (Nos. 2-11, 2-12, 1/G10, 1/G7), may be identified to be referred to three separate branches. Distribution of isolates by clusters is obviously not to correspond to their geographical origin. Besides, distribution of sets in cluster 1 did not depend on cultivar where the pathogen was isolated. In spite of the fact that a group of sets (Nos. 1/G3, 1/G4, 1/G5, 1/G6, 1/G2) taken from Gala in garden varieties of ZAO Experimental Production Farm Tsentralnoe was formed in cluster 2, three other sets from this variety were included to other clusters. Sets Nos.1/G4, 1/G5 и 1/G6 are likely to be presented by clones as they were united at minimum genetic distance (due to identical allelic set by studied SSR markers). Groups of monospore isolates from Golden Delicious (No. 1/GD) and Renet Simirenko (No. 1/RS) varieties formed no separate clusters either.

Therefore, high indicators of genetic diversity in the analyzed sampling of monospore isolates of *V. inaequalis* at low interpopulation differences may testify to a free gene flow between the populations studied stipulated (as they are too distant from each other to allow natural transfer of spores) by human activity that is agreed with information from references [10, 11, 15]. SSR markers have revealed genetic diversities between populations of pathogen having been formed in agrophytocenoses different in structure: allelic polymorphism of SSR locus in *V. inaequalis* was higher in heterogenetic collection trees (sampling point No. 2) than in single-cultivar planting of industrial orchards (likely due to far more high variety of host plant). High level of genetic diversity in phytopathogen population is known to prevent from domination of single supervirulent and aggressive biotypes thereby decreasing probability of epiphytoties occurrence [28]. This fact is proving the approach based on application of plantings with mixed varieties.

It should be noted that there are some differences in the results of assessing polymorphism both in our research of North Caucasus population and in papers where European *V. inaequalis* populations are considered. The reason for this is likely to be vast genetic distances between *V. inaequalis* populations in the indicated regions which may testify both to limitation of gene flow from Europe to North Caucasus and to independent formation of pathogen population in North Caucasus region. One of the factors for such formation may be explained that North Caucasus is situated within the area of *M. orientalis* species, i.e. host plant for *V. inaequalis*.

Thus, the obtained results suggest significant genetic diversity in the investigated set of monospore isolates of *Venturia inaequalis*. Clusterization of isolates does not depend on geographical origin thereof, thereby indicating low inter-population differences. At the same time clusterization is closely related with planting type (single-cultivar orchards or heterogeneous collection varieties) but not always depends on cultivar where isolates were collected. Polymorphism observed for some SSR markers corresponds to that described on European populations of pathogen, but there are also some differences that in our opinion could be due to peculiarities of formation of North Caucasus pathogen population.

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SEED CARBOHYDRATE COMPOSITION AND ITS RELATION TO ANOTHER BREEDING IMPORTANT TRAITS OF GARDEN PEA (*Pisum sativum* L.) IN KRASNODAR REGION

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Abstract

Starch is the main pea (*P. sativum* L.) seed carbohydrate in which the amylose to amylopectin ratio is controlled genetically. Recessive allele *r* of locus RUGOSUS determines an increase in the amylose fraction which leads to the wrinkled-seed character of vegetable peas. A high proportion of amylose in pea starch (more than 70 %) promotes a slow transition of sugars to starch and results in a longer period of technical ripeness. High-amylose starch determines the use of peas in dietary nutrition, and also as raw materials for biodegradable plastics and films. Pea diversity on amylose content is poorly studied, and no data about the relationship between this trait and other agronomically valuable parameters is available. This study is the first to report polymorphism of VIR Collection accessions (Vavilov Institute of Plant Genetic Resources, St. Petersburg, Russia) and breeding forms from Krymsk Experimental Breeding Station (Krasnodarskii krai, Russia) on carbohydrate composition under contrast weather conditions, and a relationship between the amylose content in seeds, smaller seed size and seed number per pod. The objectives of this work were to reveal garden pea polymorphism on starch carbohydrate composition, to seek for genotypes with high-amylose starch in mature seeds and to estimate the relationships between the seed starch composition and other valuable traits in pea plants. In 2015 and 2016, 39 vegetable pea specimens were tested in field trials in Krasnodarskii krai. Starch content in seeds was determined polarimetrically, and iodine-based calorimetry was used for amylose assessment. The biochemical traits were influenced by environmental conditions. In more favorable 2016 as compared to 2015, seed productivity per plant was 4.5 g higher, the starch accumulation decreased by 3.6 %, whereas the amylose content in starch increased by 1.6 % ($p < 0.05$). There was no statistically significant difference in amylose content detected between accessions of leafy (*Af*) and semi-leafless (*af*) morphotypes in contrast weather conditions of 2015 and 2016 which indicates that both types may be involved in breeding programs. In both years, the highest amylose proportion in starch was in accessions with smaller 1000 seed weight ($r = –0.34$ in 2015, $r = –0.32$ in 2016; $p < 0.05$) and the largest seed number per pod ($r = 0.47$ in 2015, $r = 0.41$ in 2016; $p < 0.05$). Starch and amylose contents correlated inversely ($r = –0.60$ in 2015, $r = –0.49$ in 2016; $p < 0.05$). Varieties Grundy, Durango, and Gropesa were starch-rich with the high amylose level in starch and could serve as donor genotypes. Starch and amylose contents, as averaged over a two-year period, were 31.9 and 75.1 % ($p < 0.05$), respectively, for Grundy, 32.0 and 74.1 % ($p < 0.05$) for Durango, 35.1 and 75.4 % ($p < 0.05$) for Gropesa. Among the varieties studied, SV 0987 UC, Vinco, Omega, Gropesa, and Butana possessed the high amylose level in seeds (79.5, 72.2, 74.0, 75.4 and 72.4 %, respectively, as averaged over 2 years) and small-sized seeds ($p < 0.05$). Varieties Mucio and Olinda were high in amylose (77.5 and 71.4 %, respectively) in combination with more than 8 seeds per pod ($p < 0.05$).

Keywords: starch, amylose, seed productivity, morphotypes, *Pisum sativum* L., garden peas, relations of the traits

Vegetable pea (*Pisum sativum* L.) is the most important food crop being a

source of high quality proteins, carbohydrates, micronutrients and vitamins in its technical ripeness stage. Main form of carbohydrates in pea seeds is presented by starch. Polysaccharide ratio of amylose to amylopectin ratio forming the starch is controlled genetically. At least 60% of amylose is known to be accounted for pea starch with wrinkled seed character (marrowfat pea) typical to vegetable pea variety, while 25-30% — to smooth-seeded pea [1]. It is similar to cellulose (which is very important in supporting bowel action thereby preventing colon cancer) in nature of monomers from which starch polysaccharide is made of, i.e. α-form of glucose in starch and β-form in cellulose, while structure of homopolymers to be formed is not the same [2-4].

High amylose starch identifies slow overripenning of seeds at technical ripeness stage [5], which is required in vegetable pea processing. High amylose starch is also considered as valuable stock in chemical industry when manufacturing biodegradable plastics [6], e.g. technical and packing (including edible packing for food industries), as well as thermoplastic films for medicine [7]. Thus, development of vegetable pea cultivars, with seeds thereof containing larger content of both vitamins, micronutrients and amylose, is becoming the most important direction in breeding especially considering biofortification strategy i.e. increase of nutrition crop.

At least 6 genes (loci) responsible for synthesis of starch, amylose and amylopectin (*r, rb*, *rug3*, *rug4*, *rug5, lam*) [8] are described in peas. Recessive allele *r* of locus RUGOSUS is the most important one to be used for breeding. If it is in homozygous state it defines marrowfat (wrinkled) seed character. Activity of SBE (starch branching enzyme) responsible for amylopectin synthesis is greatly decreasing in *rr* genotypes in germs resulting accordingly in reducing of starch amount, increase of amylose and 2-fold sucrose accumulation as compared with similar values for smooth-seeded peas. These plant seeds are sweeter and absorb more water which they give back at ripening thereby becoming wrinkled over surface. Relative fraction of reserve protein legumin is reducing therewith [9]. Recessive mutation of *rb* gene reduces activity of ADP-Glucose Pyrophosphorylase responsible for rate of sucrose-to-starch transformation, thereby resulting in two-fold reducing of starch synthesis (as compared with wild type) and glucose and fat content increasing [10, 11]. Recessive allele *rug3* is revealed by reducing starch content both in seeds (up to 1% of dry substance, while in wild types it reaches 50%), and in leaves due to no activity of plastid phosphoglucomutase (PGM) catalyzing transformation of glucose-6-phosphate to glucose-1-phosphate [12]. Mutation in locus *rug4* results in reducing of sucrose (Sus) synthetase activity in growing seeds, roots, tubercules and leaves [13]. Recessive allele *rug5* results in reducing of starch synthase activity II (SSII) involved in amylopectin synthesis by elongating molecules thereof as compared to the molecules of wild type [14]. Allele *lam* is encoding relaxed version of starch synthase I related to granules, affecting neither seed shape change, not starch content thereby resulting in great changing of amylose synthesis in embryos [15, 16]. Amylose content in starch is known to be increased in final stage of seed growth [17].

There are few papers in the world scientific literature devoted to screening of the pea varieties by amylose content in seeds and by type of this trait variation [2, 3, 18, 19]. M. Hybl et al. [19] in spite of large volume of the material analyzed (over 400 samples) have made evaluation within one year and they think the achieved results as preliminary ones. Therewith, there is absolutely no information on relation between vegetable pea starch composition and other traits important for breeding. Besides, more leafless morphotypes (*af*) and forms with determinant growth type (*det, def*) with vast changes in morpho-physiological plant state are involved in vegetable pea breeding [20]. Formation of leafless high

amylose pea variety was reported, with starch content in seeds being 28.8% and 70.9% amylose in starch [21]. Generating of forms with stem fasciation combined with determinant growth type (*det* and *fa* gene alleles) is promising [22-24]. Therefore, to plant high amylose varieties possessing the above traits one should know relations between such traits and carbohydrate composition of seeds.

For the first time in this paper we have studied polymorphism of the vegetable peas samples set taken both from VIR collection and breeding forms by starch composition in ripened seeds having compared the figures in vegetation seasons at various weather conditions, and we have identified genotypes with high amylose content in starch. Therewith we have found relation between amount of amylose, smaller seed size (low weight of 1000 seeds) and amount of seeds per pod, and saw no differences in amylose accumulation between traditional morphotypes and the ones obtained under mutations thereby indicating suitability of both usual and leafless forms in breeding high amylose varieties.

This paper is devoted to quantitative evaluation of composition of starch and amylose in vegetable pea seeds and analysis of relations between this value and other plant traits important for breeding purposes.

Techniques. A total of 39 samples of vegetable peas from VIR collection (Vavilov Institute of Plant Genetic Resources) and breeding forms from Krymsk Experimental Breeding Station were taken to be analyzed. The set of samples included current varieties, perspective lines and original mutant forms from six countries: Russia (18 accessions), the Netherlands (16 accessions), Germany (2 accessions), Belgium (1 accession), USA (1 accession) and Turkey (1 accession). Seeds from all samples are marrowfat (wrinkled) suggesting availability of recession allele *r* in homozygous state in genotype. Thirty samples out of 39 possessed usual morphotype by leafy (*Af*) while 9 were leafless (no leaves) (mutation *af*). Three samples therewith were homozygotes by allele *det* regulating limited (determinant) growth type. Besides, the sampling included original fasciated form (spontaneous mutant by *fa* or *fas* alleles).

Field survey took place in 2015-2016 in seed-trial grounds of Krymsk Experimental Breeding Station (Krasnodar Territory). Soil of the ground included argillaceous compact and degraded chernozem. Seeding was made by planter SKS-6-10 with 15 cm row width and 6 cm in depth, and plot area for each sample was 10 m^2. According to vegetable pea seeding rate the plot includes 900-1200 plants from which 15 plants in the technical ripeness stage have been taken for morphostructural analysis. At biological ripeness the seeds have been harvested by Sampo130 combine (Sampo Rosenlew Oy, Finland). After the seeds reach optimum humidity (14%), the samples have been taken for biochemical analysis. Phenological, morphological and biometric parameters have been analyzed as per recommendations [25] and field test procedure [26]. Bundles to be accounted have been gathered for evaluating plant productivity at technical ripeness stage. Plants in bundles have been hand threshed, with the obtained green pea being weighed afterwards, and weight of one plant seed has been calculated.

Starch content by Ewerse was measured using rotary polarimeter SM-3 (Zagorsk Optical Mechanics Plant, Russia) [27], amylose content in starch — by a photocolorimeter (KFK-2MP, Zagorsk Optical Mechanics Plant, Russia) [27, 28]. Samples obtained have been triple analyzed biochemically.

Average (*M*) and standard errors of mean (±SEM) have been calculated. Average values of usual and mutant forms have been compared by Student's *t*-criterion (*t*-test). Differences are considered significant at $p < 0.05$. Analysis of variance (ANOVA) has been conducted by the least significant difference test (LSD test). Statistica10 (StatSoft, Inc., USA) software has been applied for mathematical data processing.

Results. Conditions of plant vegetation during the analyzing period have been harsh. In 2015 hydrothermal index (GTI) was 7.9 (optimum value is 1.0-1.2) for planting to seadling stage. During 2-3-leaf/blooming stage, the average daily temperature exceeded optimum temperature with actually no precipitations, but as at May 31, 45.5 mm precipitations came down resulting in the fact that completion of pod formation coincided with water saturation conditions (GTI from 2.0 to 2.8). In 2016 after planting and till 2-3-leaf stage amount of effective temperatures (over 10 °C) exceeded 2015 value making favorable conditions for plant growth. During 2-3-leaf/blooming stage in 2016 amount of effective temperatures was lower, while precipitation amount was higher than in 2015, with GTI being equal to 0.8. During pod formation effective temperatures (over 10 °C) and a lot of precipitations have positively affected seed productivity from plant to technical ripeness stage.

Comparing weather conditions for the last 2 years of monitoring it may be said the second year was more favorable for vegetable pea growth and formation. Seed productivity (seed weight per plants) in technical ripeness stage in varieties and lines in 2015 was statistically far lower ($p < 0.05$) than in 2016 (by 4.5 g/plant). In 2015 productivity was varying from 3.5 to 12.9 g/plant, in 2016 — from 5.0 to 18.3 g/plant. On the contrary, average content of starch in biologically mature seeds was far higher ($p < 0.05$) in 2015 and depending on cultivar, was varying from 29.4 to 37.4%, while in 2016 — from 26.0 to 35.8%. Amylose content and variations thereof were almost the same within two years (from 61.2 to 82.1% in 2015 and from 61.9 to 82.6% in 2016) but average value in 2015 as compared with 2016 was lower by 1.6% ($p < 0.05$) (Table 1).

1. Productivity and biochemical indicators in analyzed set of varieties, lines and mutant forms of vegetable pea (*Pisum sativum* L.) by years of monitoring ($n = 39$, $M \pm$SEM, Krasnodar Territory)

Name, number in VIR catalogue (genotype)	Country of origin	Productivity, g per plant		Content, %			
				starch		amylose in starch	
		2015	2016	2015	2016	2015	2016
G-9349/5, k-9349	Russia	5.9	16.2	33.7	29.5	66.4	72.9
Uvertura, i-148154	Belgium	3.7	14.5	36.6	31.9	61.3	73.6
Salinero, i-148155	Netherlands	4.8	15.3	30.2	29.6	71.5	72.2
Asana, i-148158	Netherlands	3.5	12.0	29.6	29.6	73.8	72.7
Prim, i-0155213	Russia	5.0	12.9	32.0	27.1	70.0	70.0
Stile, i-148163 (*afaf*)	USA	4.9	10.1	31.8	30.1	67.7	72.8
Karina, i-630921	Netherlands	4.2	14.1	34.2	30.2	62.8	76.1
Khesbana, i-148159 (*afaf*)	Netherlands	7.6	9.9	33.6	29.6	68.6	75.0
Vinco, i-148164	Netherlands	5.8	8.6	31.2	29.8	68.5	75.9
Alfa 2, k-7071	Russia	9.6	7.2	31.4	28.8	68.0	71.4
Olinds, i-630922	Netherlands	6.6	17.2	34.0	31.7	70.1	72.6
G-9424/7, k-9424	Russia	8.4	14.7	36.1	31.8	69.1	74.3
Gropesa, k-9730 (*afaf*)	Netherlands	5.5	16.1	34.4	35.8	76.3	74.4
G-305/28	Russia	8.9	18.3	35.5	28.1	61.2	72.6
CB 0987 UT, к-9728	Netherlands	6.7	9.4	30.3	32.2	82.1	76.8
Grundy, i-148165	Netherlands	6.8	12.5	34.2	29.6	74.4	75.7
Mutsio, i-148166	Netherlands	8.3	8.7	32.4	29.3	75.6	79.3
Berkut, k-8856	Russia	11.6	11.0	33.8	29.5	67.5	74.6
Eshton, i-148174	Netherlands	6.0	15.1	31.9	27.9	74.3	82.6
Durango, i-148170	Netherlands	6.3	9.4	33.2	30.7	75.8	72.3
Westa, k-9352	Russia	12.5	15.7	32.7	30.8	68.1	74.0
Resal, i-148175	Netherlands	9.0	12.3	32.6	30.9	67.8	66.7
Donana, i-148177 (*afaf*)	Netherlands	7.5	5.0	32.7	32.0	66.5	64.3
Bingo, i-148178 (*afaf*)	Netherlands	9.1	12.2	34.8	31.6	70.1	68.0
Omega, i-148176	Turkey	10.1	9.4	32.4	30.2	73.0	75.0
Druzhny, k-9351 (*detdet*)	Russia	10.0	14.6	37.4	31.8	66.8	64.2
Spontanny mutant (*fafa*,*fasfas*)	Russia	10.5	12.4	34.3	34.2	65.2	63.5
G-388/45	Russia	10.7	15.9	35.1	31.1	64.7	66.8
Rayner, i-148181	Germany	12.0	15.0	34.8	34.6	68.0	62.4
Ambassador, i-148179	Germany	7.9	12.0	33.4	31.2	69.6	72.4
Adagumsky, k-7071	Russia	9.3	8.7	36.3	31.7	66.8	64.9
G-349/442 (*afaf*,*detdet*)	Russia	7.4	13.8	32.7	32.8	72.8	67.7

Table 1 continued

Parus, k-9350 (*afaf*)	Russia	8.1	11.2	30.3	26.0	75.0	78.4
G-344/16	Russia	10.2	12.2	31.3	30.4	68.7	61.9
Butana, i-148180 (*afaf*)	Netherlands	8.9	9.6	31.8	29.9	73.0	71.8
G-387 (*afaf, detdet*)	Russia	7.8	14.2	33.3	32.2	72.0	69.5
G-359/58	Russia	11.5	17.4	30.6	29.6	73.3	67.7
Krasavchik, k-9449	Russia	11.3	15.7	29.4	27.6	78.3	77.2
Istok, k-9353	Russia	12.9	11.8	32.5	30.9	74.4	76.8
M±SEM		8.1±0.4*	12.6±0.5*	33.0±0.3*	30.6±0.3*	70.2±0.7	71.8±0.8

* * Differences by years are statistically significant by Student's *t*-test at $p < 0.05$.

Comparison of seed productivity, starch content and composition in seeds of 35 samples with usual growth type but having different leafy morphotype by Student's *t*-criterion has testified that there are no significant difference (at $p < 0.05$) in manifestations of these traits between leafy (usual) and leafless (with no leaves) forms (Table 2). On average by years, productivity of leafy morphotypes was equal to 10.6 g/plant, starch content in seeds — 31.6%, amylose content in starch — 71.4%, while for leafless forms these values were 9.0 g/plant, 31.7% and 71.6% (at $p < 0.05$), respectively.

2. Seed productivity, starch content in seeds and amylose in starch in analyzed samples of vegetable pea (*Pisum sativum* L.) depending on morphotype by years of monitoring (*M*±SEM, Krasnodar Territory)

Morphotype	*n*	Seed weight per plant, g		Content, %			
				starch in seeds		amylose in starch,	
		2015	2016	2015	2016	2015	2016
Leaf type							
Usual	28	8.2±0.5	13.0±0.6	32.9±0.4	30.2±0.3	70.2±0.9	72.6±0.9
Leafless	7	7.4±0.6	10.6±1.3	32.8±0.6	30.7±1.1	71.0±1.4	72.1±1.8
Stem growth type							
Usual	35	8.0±0.4	12.5±0.5	32.9±0.3	30.3±0.3	70.4±0.8	72.5±0.8
Determinate	4	8.9±0.8	13.8±0.5	34.4±1.0	32.8±0.5*	69.2±1.9	66.2±1.4*

N o t e. For description of varieties (VIR collection, Vavilov Institute of Plant Genetic Resources, Saint-Petersburg) and breeding samples (Krymsk Experimental Breeding Station, Krasnodar Territory) see Table 1.
* Differences with usual type are statistically significant by Student's *t*-test at $p < 0.05$.

It should be mentioned that over several decades the general direction in grain pea breeding is based on using leafless genotypes as being more technological and adaptive. By environmental sustainability the best leafless varieties are highly competitive with leafy ones demonstrating advantages in crop yield in years contrasting by hydrothermal regime [22]. However, they are still to be considered for vegetable pea breeding in our country. Meanwhile earlier in Krasnodar Territory we have found vegetable varieties of leafless morphotype that are reliably similar in crop to standard varieties of usual morphotype [29] by green pea crop, dry matter distribution, productivity per leaf area and net productivity of photosynthesis. These results make us sure that high amylose varieties may be formed from leafless morphotypes.

Practical application of mutant forms with restricted stem growth is still to be investigated. In 2015 no certain peculiarities and significant differences in productivity and carbohydrate composition in seeds of determinate samples and stem faciation forms as compared with average values in sampling of plants with usual stem growth type was found either (see Table 2). However, in 2016 higher starch content in seeds and lower of amylose in starch as compared with the samples bearing no such mutations was found (see Table 2). On average over the years mutant forms productivity was 11.3 g/plant, starch content in seeds 33.6%, and that of amylose in starch 67.7% ($p < 0.05$). Varieties with determinate growth type are known to be technological one at cropping and they are characterized by reduced reproductive period, good ripening, and standing ability [21, 23]. Therefore, we consider that research of carbohydrate composition in such mutant seeds is to be analyzed further with more samples.

Thus for two years of observation starch content in all analyzed samples of vegetable pea was changing within 26.0-37.4% range, and amylose from 61.9 to 82.6%. Such large variability requires gene pool screening of vegetable pea when searching for original material for breeding high amylose varieties.

3. Correlations between content of amylose and starch in seeds and other traits important for breeding in analyzed samples of vegetable pea (*Pisum sativum* L.) by years of monitoring (Krasnodar Territory)

Trait / Trait	1	2	3	4	5	6	7	8	9	10	11	12
						2015						
1	1.00	0.31	0.92*	0.21	0.41*	0.78*	0.67*	0.24	0.15	0.67*	0.00	0.21
2		1.00	0.24	0.06	0.02	0.03	0.44*	0.61*	–0.37*	–0.22	0.08	–0.34*
3			1.00	0.15	0.37*	0.67*	0.61*	0.25	0.20	0.61*	–0.01	0.26
4				1.00	–0.61*	0.39*	0.12	–0.10	–0.07	0.29	–0.06	–0.09
5					1.00	0.41*	0.09	0.06	–0.01	0.30	0.07	0.21
6						1.00	0.37*	–0.12	0.05	0.82*	–0.02	0.19
7							1.00	0.27	0.45*	0.50*	0.07	0.08
8								1.00	–0.28	–0.30	0.16	–0.34*
9									1.00	0.59*	–0.05	0.47*
10										1.00	–0.07	0.42*
11											1.00	–0.60*
12												1.00
						2016						
1	1.00	–0.03	0.91*	0.38*	0.34*	0.41*	0.39*	–0.06	–0.29	0.17	0.24	–0.36*
2		1.00	–0.05	–0.08	–0.17	–0.62*	0.36*	0.64*	–0.35*	–0.69*	–0.38*	–0.32*
3			1.00	0.19	0.41*	0.38*	0.36*	–0.05	–0.15	0.21	0.14	–0.20
4				1.00	–0.58*	0.18	0.17	–0.23	–0.19	0.09	–0.01	–0.08
5					1.00	0.39*	0.00	–0.06	–0.04	0.26	0.29	–0.17
6						1.00	–0.04	–0.56*	0.12	0.88*	0.42*	–0.04
7							1.00	0.17	0.29	0.07	–0.12	–0.11
8								1.00	–0.26	–0.62*	–0.18	–0.23
9									1.00	0.57*	–0.06	0.41*
10										1.00	0.32*	0.17
11											1.00	–0.49*
12												1.00

Note. 1 — seadling/technical ripeness stage, days; 2 — weight of 1000 seeds, g; 3 — amount of nonproductive nodes, unit; 4 — amount of productive nodes, unit; 5 — number of pods per seedstalk, unit; 6 — number of filled pods per plant, units; 7 — pod length, cm; 8 — pod width, cm; 9 — number of seeds per pod, unit; 10 — seeds from plant, unit; 11 — starch content in seeds, %; 12 — amylose content in starch, %.
* Correlations are statistically significant at $p < 0.05$.

Correlation analysis (Table 3) has revealed stable relationship within two years. Starch and amylose contents correlated inversely ($r = –0.60$ in 2015, $r = –0.49$ in 2016). It should be mentioned that scientific literature information on correlation between amylose and starch content in wrinkled seeds is contradictory. R.Kosson et al. [30] indicate negative correlation, while M. Hybl et al. [19] discovered positive relationship between these traits. However, the latter consider their results preliminary, and they also think such screening is to be used for breeding in improving carbohydrate composition in seeds of vegetable pea. We were the first in finding negative relationship between amylose content in starch and 1000 seed weight ($r = –0.34$ in 2015, $r = –0.32$ in 2016) and positive with the seed number per pod ($r = 0.47$, $r = 0.41$ over years). I.e. the less is weight of 1000 seeds, the more is their number per pod and the higher is amylose content in starch, and higher starch content in seeds suggested decrease of amylose content. Therefore, the following samples with amylose content in starch exceeding 70% and weight of 150 g per 1000 seeds may be considered promising varieties for breeding: SV 0987 UT (k-9728, 87g), Vinko (i-148164, 131g) and Omega (i-148176,135g) of traditional morphotype, leafless varieties of Gropesa (k-9730, 87g), Butana (i-148180, 146g) and varieties with 8-10 seeds per pod Mutsio (i-148166, 10.0 seeds), Grundy (i-148165, 8.5 seeds), SV 0987 UT (8.4 seeds), Butana (8.4 seeds), Olinda (i-630922, 8.3 seeds), Gropesa (8.0 seeds). Varieties Mutsio and Olinda are noted to be characterized by average variation of number of seeds

per pod for two years of monitoring (*Cv* from 10 to 20%), while this number was higher for the others.

For two years of monitoring we have chosen 15 samples out of 39 with high amylose content in starch (over 70%) (Table 4). Varieties significantly exceeding the control variety by starch content in seeds have also been found/ These are Grundy with average starch content of 31.9% and amylose in starch of 75.0% by years, Durango with 31.8 and 74.1%, respectively, and Gropesa of leafless morphotype with 35.0 and 75.4% (see Table 4).

4. Vegetable pea varieties (*Pisum sativum* L.) with high content of starch and amylose among 39 analyzed samples (by years of monitoring, Krasnodar Territory)

Variety	Country of origin	Content, %			
		starch		amylose in starch	
		2015	2016	2015	2016
Prima (C)	Russia	32.2	27.1	70.0	70,0
Salinero	Netherlands	30.3*	29.6*	71.5	72,2
Asana	Netherlands	29.6*	29.6*	73.8*	72,7*
Olinda	Netherlands	33.8*	31.7*	70.1	72,6*
Gropesa[a]	Netherlands	34.2*	35.8*	76.3*	74,4*
SV 0987 UT	Netherlands	30.1*	32.2*	82.1*	76,8*
Grundy[a]	Netherlands	34.1*	29.6*	74.4*	75,7*
Mutsio	Netherlands	32.1	29.3*	75.6*	79,3*
Eshton	Netherlands	31.8	27.9*	74.3*	82,6*
Durango[a]	Netherlands	32.9*	30.7*	75.8*	72,3*
Omega	Turkey	32.3	30.2*	73.0*	75,0*
Parus	Russia	30.4*	26.0*	75.0*	78,4*
Butana	Netherlands	31.8	29.9*	73.0*	71,8
Krasavchik	Russia	29.4*	27.6	78.3*	77,2*
Istok	Russia	32.3	30.9*	74.4*	76,8*
M±SEM		31,8±0,4	29.9±0.6	74.5±0.8	75.2±0.9

N o t e. C — control variety; [a] — sources of alleles with high content of starch in seeds and amylose in starch. For description of varieties (VIR collection, Vavilov Institute of Plant Genetic Resources, Saint-Petersburg) and breeding samples (Krymsk Experimental Breeding Station, Krasnodar Territory) see Table 1.
* Differences from control are statistically significant at $p < 0.05$.

So, the dependence of carbohydrate composition of vegetable pea seeds (amount of starch in seeds and amylose content therein) on vegetation conditions is found, and relationship between these biochemical parameters and number of trait important for plant breeding is revealed. Both amylose content in seed and weight of 1000 seeds, as well as amount of starch in seeds and amylose content in starch correlate negatively; whereas the correlation between amylose content in starch and number of seed per pod is positive. No statistically significant differences by seed productivity, starch content in ripened seeds and amylose in starch between leafy (usual) and leafless (with no leaves) morphotypes are found. Consequently, breeding with higher content of starch and amylose in seed is possible with the use of both morphotypes. Samples that may be used in breeding as gene sources identifying high content of starch in seeds and amylose in starch are revealed. Traits positively correlating with high accumulation of amylose, i.e. small-sized seeds (weight of 1000 seeds less than 150 g), and number of seeds per pod over 8, are also found.

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PRODUCTION OF TERMINALLY N-DEACETYLATED OLIGOMERS OF CHITOSANE USING RECOMBINANT CHITOOLIGOSACHARIDE DEACETYLASE NodB OF BACTERIA *Mesorhizobium loti* EXPRESSED IN *Escherichia coli*

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Abstract

Chitin and chitosan oligomers affect the growth and development of plants and are able to induce plant resistance to infection with phytopathogens, which determines the interest in the preparation and use of these compounds. The influence of chitosan oligomers on the plant directly depends on the degree of deacetylation, but it is difficultly to obtain compounds with necessary structure using hydrolysis of the polymer or chemical synthesis. Such problems can be solved in the process of biosynthesis of chitooligosaccharides, when enzymes with specific activity are used. The selectivity of the chitooligosaccharide deacetylase (EC 3.5.1.-) of rhizobia to carry out the monodeacetylation of the chitooligosaccharides at the terminal position of the molecule causes interest in studying the possibility to use this enzyme for the synthesis of such compounds. In current work we have developed approaches for the synthesis of mono-deacetylated chitopentaose (tetra-N-acetylchitopentaose) using *Mesorhizobium loti* CIAM1026 enzyme chitooligosaccharide deacetylase. Heterologous expression of the *nodB* gene encoding the *M. loti* chitooligosaccharide deacetylase in *Escherichia coli* XL1-Blue MRF' and SHuffle express strains using the modified pOPE101mod-*nodB* vector with deleted *pelB* sequence resulted in soluble enzyme preparation. The amount of soluble enzyme was higher in SHuffle express strain, which was specially developed for correct formation of disulfide bonds in synthesized proteins. Studying the properties of the enzyme purified on Ni-NTA agarose showed its ability to deacetylate penta-N-acetylchitopentaose at the terminal position. Mass spectrometric analysis confirmed the use of practically the entire substrate for the preparation of deacetylated tetra-N-acetylchitopentaose. Methods for the separation and purification of deacetylated chitooligosaccharides by ion exchange chromatography followed by desalination have been developed. Synthesis of terminally N-deacetylated chitosan oligomers may be a necessary step in the preparation of their conjugates with biologically active compounds.

Keywords: chitin and chitosan oligomers, *Mesorhizobium loti* chitooligosaccharide deacetylase, pOPE101-215(Yol) and pRSETb vectors, biosynthesis, *Escherichia coli* SHuffle express and XL1-Blue MRF'

Chitin and chitosan oligomers (chitooligosaccharides) are widely used in medicine and agrotechnologies. In agriculture, interest in these compounds is associated with their ability to induce nonspecific resistance of plants to infection by phytopathogens due to the activation of the natural protective potential (elicitor activity) [1, 2], as well as to stimulate the growth and development of plants. The specific feature of chitooligosaccharides when used as regulators is non-toxicity even in significant quantities and ease of disposal, which makes them environmentally safe [3-5].

For the elicitor activity to manifest itself, the degree of deacetylation of chitooligosaccharides is important, because in case of some plant species (arabidopsis, rice, wheat), eliciting properties are shown by fully acetylated chitin oligomers, in case of others (peas, coffee, parsley), by partially or completely deacetylated chitosan oligomers [6-9]. In addition, deacetylation of chitooligosaccharides in certain positions may allow various chemical groups to covalently attach to them, which will significantly expand the range of such compounds.

In chemical synthesis, it is difficult to obtain compounds with the necessary degree of deacetylation. In particular, it is almost impossible to synthesize partially deacetylated chitin oligomers in this way due to the identical chemical activity of the amino groups in sugar residues, which does not allow the deacetylation reaction to be controlled due to harsh conditions. Similar problems can be solved during the biosynthesis of chitooligosaccharides, when enzymes with selective specificity are used and, as a result, products with a strictly defined structure are formed. In this connection, the search and study of enzymes involved in the synthesis of chitin oligomers and their deacetylated derivatives are of great interest.

In *Rhizobium* spp. bacteria, the *nodABC* genes [10-13], which are common for all rhizobial species, encode enzymes necessary for synthesizing the core structure of Nod factors, the signal molecules that control the development of legume-rhizobial symbiosis. NodC is an N-acetylglucosaminyltransferase catalyzing the synthesis of chitin oligomers. Chitooligosaccharide deacetylase NodB is required for deacetylation of chitooligosaccharide at the non-reducing end (terminal N-acetylglucosamine) [14], which allows fatty acid to be attached to it using NodA acyl transferase. The selective ability of rhizobial chitooligosaccharide deacetylase to carry out monodeacetylation at the terminal position determines the interest in studying the possibility of using this enzyme to synthesize deacetylated chitosan oligomers in vitro.

Previously, we developed an approach to obtain chitin oligomers associated with the use of the N-acetylglucosaminyltransferase enzyme of two strains of rhizobial bacteria — *Rhizobium* sp. GRH2 and *Mesorhizobium loti* CIAM1803 [15]. In this study, we carried out heterologous expression of the *nodB* gene encoding *M. loti* CIAM1803 chitooligosaccharide deacetylase in two strains of *Escherichia coli* XL1-Blue-MRF' and SHuffle express. In this case, the plasmid pOPE101-215(Yol) was used, which allows the synthesized protein to transfer to the periplasmic space thanks to the inclusion in the pelB leader sequence, which ensures its safety and correctness of folding [16]. This plasmid was successfully used to synthesize the heavy and light chains of immunoglobulin G [16]. Earlier, an attempt was made to synthesize NodB of *Sinorhizobium meliloti* in *E. coli* BL21 (DE3) bacteria using the pET-3c vector, while the recombinant protein formed inclusion bodies, which required refolding (probably, therefore, the enzyme activity was low) [14]. An active soluble protein NodB of *Rhizobium* sp. GRH2 has been recently obtained by a group that synthesized it in BL21(DE3) cells transformed with the pET22b(+) vector [17], but the enzyme yield was not high enough.

In this work, using the pOPE101-215(Yol) vector and its modified version pOPE101mod with the *pelB* sequence removed, used to transform two strains of *E. coli* (SHuffle express and XL1-Blue MRF'), we managed to achieve a high yield of soluble protein, the NodB *Mesorhizobium loti* chitooligosaccharide deacetylase enzyme capable of deacetylating penta-N-acetylchitentaose in terminal position. Methods for selective extraction of the formed deacetylated chitooligosaccharides have been also developed.

The work aimed to study the possibility of obtaining terminally N-deacetylated chitosan oligomers using NodB chitooligosaccharide deacetylase, synthesized by heterologous expression of specially designed gene constructs.

Techniques. DNA was isolated from the *Mesorhizobum loti* CIAM 1803 strain (WDCM 966). The *E. coli* DH5α strain was used for standard cloning procedures. The synthesis of proteins was carried out in a mutant strain of *E. coli* C41 obtained on the basis of BL21(DE3) [18], as well as in strains XL1-Blue MRF' (Stratagene, USA) and SHuffle express (New England BioLabs, United Kingdom). *E. coli* strains were cultured in a liquid medium LB [19] or 2xYT (Difco, Netherlands) on a Heidolph Unimax 2010 orbital shaker (Heidolph Instruments GmbH, Germany) at 30 °C or 37 °C with the addition of ampicillin (100 μg/ml) in the presence of 0.04 mM or 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) as an inducer of expression. The strain of rhizobia was cultured in a liquid medium TY (trypton 5 g/l, yeast extract 3 g/l, $CaCl_2$ 0.5 g/l) at 28 °C.

When creating genetic constructs for the synthesis of NodB in *E. coli* bacteria, the full-size *nodB* gene was amplified on the DNA matrix of the *M. loti* CIAM1803 (WDCM 966) strain using primers into which the BamHI and EcoRI restriction sites were introduced for cloning in the pRSETb vector (Invitrogen, USA) or NcoI and NotI for cloning in the pOPE101-215(Yol) vector (Progen Biotechnik GmbH, Germany):

*nodB*_F_BamHI — GGGGATCCGATGAGACGTCTCGATGACAG,
*nodB*_R_EcoRI — GGGAATTCTCAGTGATGTTCTGGAAGCG,
*nodB*_F_NcoI — GGCCATGGCGATGAGACGTCTCGATGACAG,
*nodB*_R_NotI — GGGCGGCCGCGTGATGTTCTGGAAGCG.

Amplification (a Thermal Cycler C1000, Bio-Rad Laboratories, USA) was performed according to the following protocol: 5 min at 95 °C; 30 cycles — 30 s at 94 °C, 30 s at 51 °C and 30 s at 72 °C).

To obtain the modified vector pOPE101mod with the *pelB* sequence removed, the following primers were used:

*nodB*_F_NcoI — GGCCATGGCGATGAGACGTCTCGATGACAG,
pOPE101mod_R_NcoI — CCCCATGGCGGTTAATTTCTCCTCTT.

All vectors contained a His_6 sequence allowing the purification of recombinant proteins using metal chelate affinity chromatography.

The constructs were introduced into *E. coli* cells using electrical or chemical transformation methods [15, 20, 21]. Plasmid DNA was isolated from 3 ml culture using alkaline lysis [22].

To synthesize NodB protein, *E. coli* C41, XL1-Blue MRF' or SHuffle express cells were cultured in a liquid medium LB or 2xYT at 37 °C to a density of OD_{600} = 0.6-0.7, then IPTG was added to a final concentration of 0.04 or 0.4 mM, and culturing continued at 30 °C or 37 °C for 3 or 24 hrs. After completion of the synthesis, the cells were held on ice for 20 min, then precipitated by centrifugation at 3,500 g (J2-21, Beckman Coulter, Inc., USA) for 15 min at 4 °C. The cell sediment was gently resuspended in 50 mM Na-phosphate buffer (pH 7.4) containing a mixture of protease inhibitors (Sigma, USA), 1 mM dithiothreitol (DTT) and DNase (1 U/ml). The cells were sonicated 3 times for 30 s at an amplitude of 10 μm (Soniprep 150 Plus, MSE, United Kingdom), at 40-

sec intervals of holding on ice, then centrifuged at 100000 g (TL-100, Beckman Coulter, Inc., USA).

Aliquots of the soluble and insoluble protein fractions were separated in a 12-15% polyacrylamide gel (PAAG) in a Tris-glycine buffer in the presence of sodium dodecyl sulfate (SDS) (25 mM Tris-HCl pH 8.3; 192 mM glycine, 0.1% SDS) by the U.K. Laemmli method [23]. For the separation, the Mini-PROTEAN 3 system (Bio-Rad Laboratories, USA) was used, with the current intensity of 15-35 mA per gel. After completion of electrophoresis, the gels were washed with deionized water and either stained in Simple Blue solution (Invitrogen, USA) according to the manufacturer's protocol, or used for Western blot hybridization. The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot semi-dry blotting device (Bio-Rad Laboratories, USA). To check the transfer efficiency, the membranes were stained with Ponce 4R, then washed 2 times for 10 min in TBS buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl), then 2 times for 15 min in TTBS buffer (50 mM Tris -HCl, pH 8.0; 150 mM NaCl, 0.05% Tween-20). Then they were incubated for 1 hr. in a blocking solution of TTBS containing 1% bovine serum albumin (BSA), then for 2 hrs with antibodies against a His_6 sequence (anti-His) conjugated with horseradish peroxidase, which were diluted in TTBS buffer with 0.5% BSA in the ratio of 1:2000. After that, the membranes were washed 2 times for 15 min in TTBS buffer, then 2 times for 10 min in TBS buffer. To demonstrate the reaction, the Clarity Western ECL Substrate, a chemiluminescent substrate (Bio-Rad Laboratories, USA) was used. The development of the reaction was analyzed on a G:BOX-CHEMI-XX9 system (Syngene, United Kingdom).

For the standard procedure for purification of the synthesized proteins containing a His_6 sequence, 100 μl of Ni-NTA (nickel-nitrilotriacetic acid) agarose (Thermo Fisher Scientific, USA) was used. Purification was carried out at 4 °C according to the protocol proposed by the manufacturer; the protein was washed from the column with 50 mM Na-phosphate buffer (pH 7.4) containing imidazole (300 mM).

In vitro deacetylation of penta-N-acetylchitentaentase using the obtained NodB protein was performed overnight at 28 °C in 100 μl of a solution containing 3-[N-morpholino] propanesulfonic acid (MOPS, 20 mM, pH 7.2), 5 μg of an enzyme purified on Ni-NTA agarose and 1 mg of penta-N-acetylchitentaose substrate. The reaction was stopped by boiling at 95 °C for 5 min, and the samples were centrifuged for 10 min at 14000 g (Mikro 22R, Hettich GmbH & Co. KG, Germany). The supernatant was dried in a vacuum evaporator (Concentrator Plus, Eppendorf, USA). Separation and purification of the obtained chitosan oligomer was performed using ion-exchange chromatography on Toyopearl-SP 650M (0.5×2 cm) (Sigma-Aldrich, USA); elution was performed with 0.2 M NaCl. A Sep-Pak C18 cartridge (Waters, USA) was used to remove salt from the eluate.

The products of the deacetylation reaction were analyzed on a Varian 902 FT/ICR MS ion cyclotron mass spectrometer (Agilent Technologies, USA) with a 9.4 T superconducting magnet. Desorption and ionization of the sample was performed using the third harmonic of the Nd:YAG laser (λ = 355 nm). The samples were dissolved in 2 μl of a 0.1% aqueous solution of trifluoroacetic acid (TFA), an aliquot (0.5 μl) was mixed on a target with an equal volume of the matrix (2.5-dihydroxybenzoic acid at a concentration of 20 mg/ml; the solvent for the matrix was a mixture of acetonitrile and 0.1% aqueous solution of TFA in the ratio of 30%:70%) and dried in air. Then the samples were subjected to laser irradiation (5 pulses per series). The molecular weight of the sample was determined by external calibration using standards.

Results. Construction of vectors that ensure the synthesis of

NodB protein in *E. coli* cells. For heterologous expression of the *nodB M. loti* CIAM1803 gene, two types of constructs were obtained — in the pRSETb vector under the control of the promoter of T7 bacteriophage and in the pOPE101-215(Yol) vector, in which the coding sequence of the gene was cloned under the synthetic promoter in the frame with the *pelB* sequence necessary for transfer of the synthesized protein to the periplasmic space.

Synthesis of chitooligosaccharide deacetylase (NodB) in the *E. coli* C41 strain using the pRSETb-*nodB* construct. A prerequisite for protein synthesis is the ability of the *E. coli* strain to stably maintain the expression vector, to be resistant to the foreign product and to ensure its stability. Mutant strains of *E. coli* C41(DE3) and C43(DE3), derived from BL21 (DE3) [18], have such properties. When the *E. coli* C41 cells transformed with the pRSETb-*nodB* construct were cultured in the presence of a 0.4 mM IPTG inducer, NodB protein synthesis with the expected molecular weight of about 25 kDa was observed (Fig. 1). NodB production was high, but at the same time, with its bulk accumulated in the insoluble fraction obtained at 3600 g, which indicated the formation of inclusion bodies (see Fig. 1). Variation of cultivation temperature and inducer concentration did not affect the increase in the yield of NodB protein in a soluble state. Similarly, earlier when using BL21(DE3) cells transformed with the pET-3c vector, in which the *nodB* gene was cloned under the T7 promoter, all the synthesized protein was in the insoluble fraction [14]. Thus, with vectors that ensure a very high level of NodB synthesis in *E. coli* cells, it cannot be obtained in a soluble state.

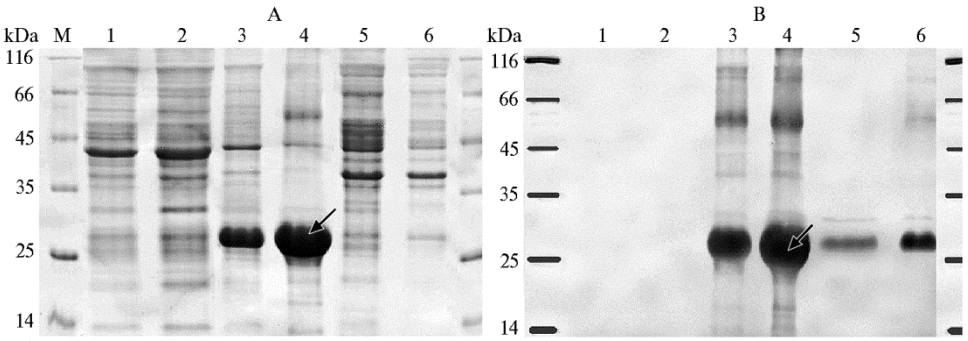

Fig. 1. Synthesis of chitooligosaccharide deacetylase (NodB) *Mesorhizobum loti* CIAM1803 in *Escherichia coli* C41 cells transformed with the pRSETb-*nodB* vector, after 20 h of culture: A — staining of Simple Blue gel, B — Western blot hybridization with anti-His antibodies. Transformation with the pRSETb vector without insert (control): 1 and 2 — cultivation without the addition of an isopropyl-β-D-thiogalactoside (IPTG) inducer and with 0.4 mM IPTG. Transformation with the pRSETb-*nodB* vector: 3 and 4 — cultivation without the addition of IPTG and with 0.4 mM IPTG (insoluble fractions obtained by centrifugation at 3600 g); 5 and 6 — culture without the addition of IPTG and with 0.4 mM IPTG (insoluble fractions obtained by centrifugation at 14000 g). M — molecular weight marker. The arrow indicates the synthesized protein.

Synthesis of chitooligosaccharide deacetylase in *E. coli* XL1-Blue MRF' and SHuffle express using the pOPE101-215(Yol) vector. To increase the yield of soluble *NodB* protein, we used the construct in the pOPE101-215(Yol) vector (Progen Biotechnik GmbH, Germany), which ensures the accumulation of protein in the periplasmic cell space. When pOPE101-215(Yol)-*nodB* was introduced into *E. coli* XL1-Blue MRF' and SHuffle express cells we observed protein synthesis with the expected molecular weight (about 25 kDa) in the presence of 0.04 and 0.4 mM IPTG. We judged this by the emergence of a product similar in motility to that synthesized in *E. coli* C41 cells when using the pRSETb-*nodB* construct. In both strains, NodB expression was

maximal when cultured overnight, at a temperature of 37 °C and an IPTG concentration of 0.4 mM (Fig. 2), but in the case of the XL1-Blue MRF' strain, NodB production was significantly higher (see Fig. 2). The protein obtained in *E. coli* XL1-Blue MRF' cells accumulated mainly in the insoluble fraction, and only a small amount of it was present in the soluble fraction (Fig. 3). In order to exclude the possibility of ineffective cell lysis when using a special buffer [16], we applied ultrasonic treatment. However, probably, due to the peculiarities of the structure of the synthesized NodB protein, we failed to detect its effective transfer to the periplasmic space. To assess the possible effect of *pelB* on the level of protein synthesis, we also obtained the pOPE101mod-*nodB* construct, from which we removed the fragment encoding this sequence.

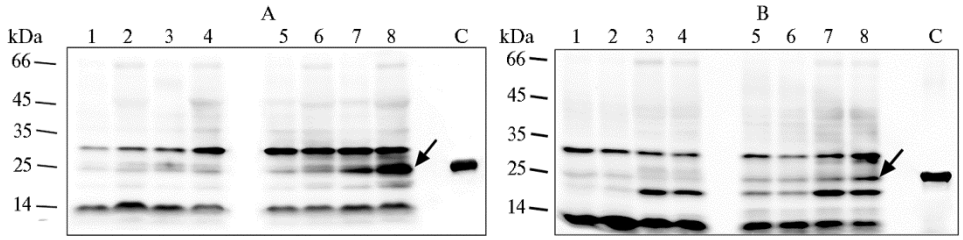

Fig. 2. Synthesis of chitooligosaccharide deacetylase (NodB) of *Mesorhizobum loti* CIAM1803 in *Escherichia coli* XL1-Blue MRF' (A) **and SHuffle express** (B) **cells transformed with the pOPE101-215(Yol)-*nodB* vector:** 1 and 2 — culture at 30 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 30 °C with 0.4 mM IPTG for 3 and 20 hrs; 5 and 6 — culture at 37 °C with 0.04 mM IPTG for 3 and 20 hrs, 7 and 8 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

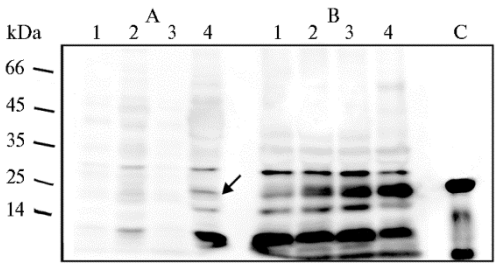

Fig. 3. Analysis of the content of NodB *Mesorhizobum loti* CIAM1803 protein in the soluble (A) **and insoluble** (B) **fractions of *Escherichia coli* XL1-Blue MRF' cells in transformation with the pOPE101-215(Yol)-*nodB* construct:** 1 and 2 — culture at 37 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

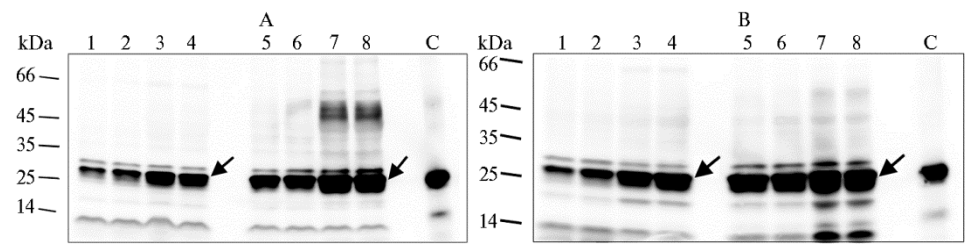

Fig. 4. Synthesis of chitooligosaccharide deacetylase NodB of *Mesorhizobum loti* CIAM1803 in transformation of *Escherichia coli* XL1-Blue MRF' (A) **and SHuffle express** (B) **with the pOPE101mod-*nodB* vector:** 1 and 2 — culture at 30 °C in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture at 30 °C with 0.4 mM IPTG for 3 and 20 hrs; 5 and 6 — culture at 37 °C with 0.04 mM IPTG for 3 and 20 hrs, 7 and 8 — culture at 37 °C with 0.4 mM IPTG for 3 and 20 hrs. C (control) — NodB protein synthesized in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs at 37 °C in the presence of 0.4 mM IPTG. Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

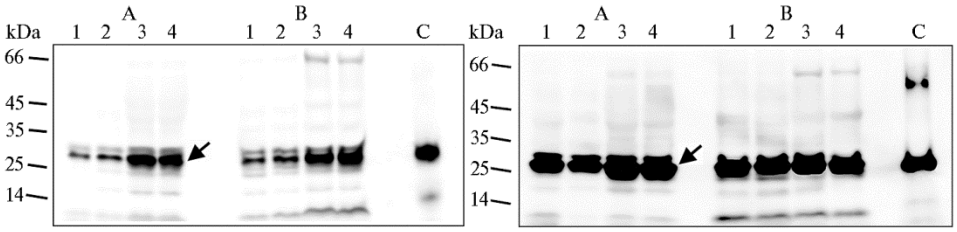

Fig. 5. The content of NodB of *Mesorhizobum loti* CIAM1803 protein in the soluble (A) **and insoluble** (B) **fractions in transformation of *Escherichia coli* XL1-Blue MRF'** (left) **and SHuffle express** (right) **with the pOPE101mod-*nodB* construct (culture at 30 °C):** 1 and 2 — culture in the presence of 0.04 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 and 20 hrs, 3 and 4 — culture with 0.4 mM IPTG for 3 and 20 hrs. C (control) — synthesis of NodB protein in *E. coli* C41 cells using the pRSETb-*nodB* vector after 20 hrs in the presence of 0.4 mM IPTG (insoluble fraction). Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

Synthesis of chitooligosaccharide deacetylase (NodB) in *E. coli* XL1-Blue MRF' and SPuffle express cells using the pOPE101mod-*nodB* vector. When using the pOPE101mod-*nodB* vector, from which the *pelB* leader sequence was removed, a higher level of NodB production was observed in two types of *E. coli* cells (XL1-Blue MRF' and Shuffle express) compared to that in the variant of transformation with the initial vector pOPE101-215(Yol)-*nodB* (Fig. 4, 5). When culture in the presence of 0.4 mM IPTG and at temperatures of 30 and 37 °C, a high level of NodB synthesis was observed already after 3 hrs in the cells of two *E. coli* strains, the XL1-Blue MRF' and SHuffle express (see Fig. 4, 5).

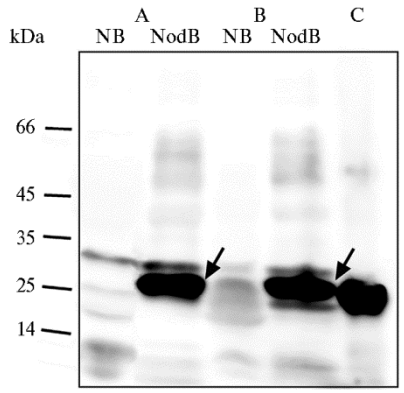

Fig. 6. Purification of recombinant protein NodB of *Mesorhizobum loti* CIAM1803 on Ni-NTA agarose: A and B — transformation of *Escherichia coli* XL1-Blue MRF' and SHuffle express strains with the pOPE101mod-*nodB* construct, C (control) — transformation of *E. coli* C41 with pRSETb-*nodB*; NB is a protein that did not bind to Ni-NTA agarose, NodB is a protein purified on Ni-NTA agarose. Western blot hybridization with anti-His antibodies. The arrow indicates the synthesized protein.

In this case, the amount of soluble protein was higher at a temperature of 30 °C and with the use of the SHuffle express strain (see Fig. 5), specially designed to ensure the correct formation of disulfide bonds in proteins [24]. The increase in the yield of the recombinant product in the soluble state as the cultivation temperature decreases can probably be determined by the fact that under such conditions the synthesis of chaperones in the cell enhances [25]. Thus, we managed to obtain chitooligosaccharide deacetylase in a soluble state.

Analysis of the products of deacetylation of penta-N-acetyl chitopentaose by the *M. loti* CIAM1803 chitooligosaccharide deacetylase enzyme. To test the enzymatic activity of the obtained chitooligosaccharide deacetylase, we studied its ability to deacetylate penta-N-acetyl chitopentaose which is the main substrate of this enzyme in *M. loti* bacteria. For this purpose, we purified the recombinant protein obtained during the synthesis in *E. coli* XL1-Blue MRF' and SHuffle express cells with the use of metal chelate affinity chromatography on Ni-NTA agarose (we obtained about 200 μg of partially purified protein from 50 ml culture) (Fig. 6). The purified enzyme was incubated with the substrate (penta-N-acetyl chitopentaose). Mass spectrometry of the samples obtained after the deacetylation reaction catalyzed by NodB

chitooligosaccharide deacetylase (Fig. 7) revealed a compound with the mass-to-charge ratio (m/z) of 1014.390. The expected mass of chitopentaose deacetylated at one position is 991. However, an H^+ or Na^+ ion usually attaches to the test substance during a mass spectrometric analysis. If Na^+ attaches, the molecular weight of the analyzed substance will be 991 + 23 = 1014. Thus, the substance to be synthesized is tetra-N-acetyl chitopentaose (monodeacetylated chitopentaose). The analysis showed that chitooligosaccharide deacetylase obtained by synthesis in *E. coli* SHuffle express and XL1-Blue MRF' bacteria has the necessary enzymatic activity.

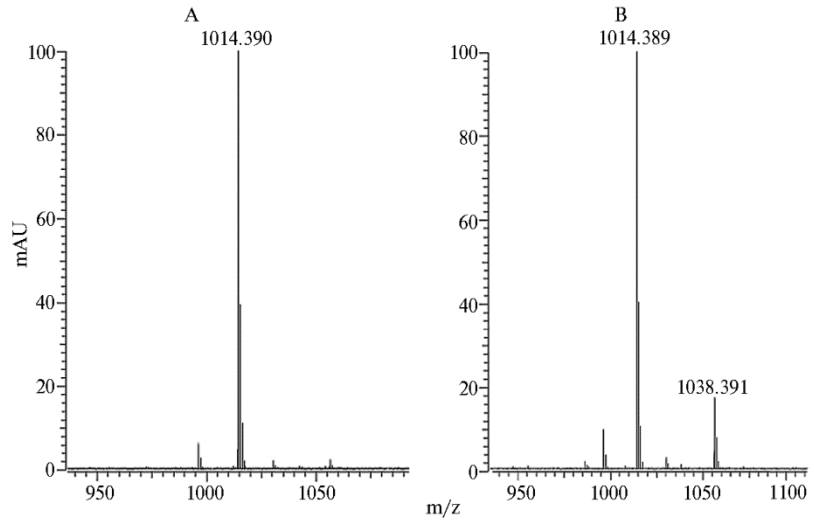

Fig. 7. Mass spectrometry analysis of the products of deacetylation of penta-N-acetyl chitopentaose by the *Mesorhizobum loti* CIAM1803 chitooligosaccharide deacetylase enzyme synthesized in *E. coli* SHuffle Express (A) **and XL1-Blue MRF'** (B) **strains with the pOPE101mod-*nodB* vector** (Varian 902 FT/ICR MS mass spectrometer, Agilent Technologies, USA).

Purification of N-terminal deacetylated chitooligosaccharides. We also investigated the possibility to separate deacetylated chitooligosaccharides (due to the presence of a free amino group in the molecule) by ion-exchange chromatography. It was necessary to solve the problem of removing the aqueous-saline eluent after chromatography. To isolate the N-terminal deacetylated chitooligosaccharide, we used ion-exchange chromatography on Toyopearl-SP 650M; elution was performed with 0.2 M NaCl. A Sep-Pak C18 cartridge was used to remove salt from the eluate. This is due to the fact that oligomers of both chitin and chitosan are able to reversibly interact in the aqueous eluent with reversed-phase sorbents, and the time of their yield from the column is several times longer than the yield of inorganic salts [26]. The chitooligosaccharide solution after ion-exchange chromatography (no more than one volume of the cartridge) was passed through a sorbent, which was then washed with water. NaCl molecules pass through a hydrophobic medium with practically no dilution effect; therefore, to achieve complete desalting, it is enough to flush the column with 3 cartridge volumes. After this, the chitooligosaccharide was eluted with water; the desalted solution was lyophilized and analyzed. Thus, we have developed methods for the selective isolation of deacetylated chitooligosaccharides.

In our work, we studied the possibility of synthesizing the chitooligosaccharide deacetylase enzyme (NodB) of *M. loti* bacteria in *E. coli* cells in a soluble state and using it to obtain terminally N-deacetylated chitosan oligomers. In soil rhizobial bacteria, chitooligosaccharide deacetylase is a soluble cytoplasmic

protein that participates in the initial stages of the synthesis of signal molecules of Nod-factors, in particular, deacetylates the chitin oligomers (n = 4-6) obtained at the first stage of synthesis at the non-reducing end [27]. The possibility of obtaining terminally N-deacetylated chitin oligomers in vitro using the chitooligosaccharide deacetylase enzyme is of practical interest, since such compounds are very difficult to obtain during chemical synthesis, but it is convenient to use them for covalent attachment of biologically active substances.

For the heterologous expression of the *nodB M. loti* gene in *E. coli* bacteria, several genetic constructs were used, including those enabling the accumulation of the synthesized protein in the periplasmic space. However, in our experiments during the synthesis in *E. coli* C41 cells with the pRSETb-*nodB* construct, as well as in XL1-Blue MRF' and SHuffle express with the pOPE101-215(Yol)-*nodB* construct, we initially failed to obtain a significant amount of NodB protein in a soluble state. Most of the synthesized protein in the cells was contained in the insoluble fraction of cells. The observed ineffective removal of NodB into the periplasmic space was probably associated with the conformational features of this protein.

However, by removing the *pelB* sequence from the expression vector, we were able to increase the production of soluble protein in the SHuffle express and XL1-Blue MRF' cells. At the same time, the quantitative yield of NodB protein after purification on Ni-NTA agarose was quite high. Testing of the activity of purified soluble NodB protein confirmed its ability to deacetylate penta-N-acetylchitentaose at its non-reducing end. Mass spectrometry analysis showed that practically the entire substrate was used to obtain deacetylated tetra-N-acetyl-chitopentaose. A similar activity was detected for another rhizobium enzyme, NodB of *Rhizobium* sp. GRH2, synthesized in a soluble state in *E. coli* BL21(DE3) cells using the construct in the pET-22b(+) vector [17].

Thus, for the first time, the proposed approach using the modified vector pOPE101mod-*nodB* allows the synthesis of *Mesorhizobum loti* active chitooligosaccharide deacetylase in a new type of *Escherichia coli* SHuffle express and XL1-Blue MRF' cells with high quantitative yield. For the purpose of separation and purification of the resulting deacetylated chitooligosaccharides, we have developed methods based on ion-exchange chromatography followed by desalting.

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ACTIVITY OF INSECTICIDAL *Bacillus thuringiensis* var. *israelensis* STRAINS STORED BY VARIOUS METHODS

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Abstract

Microbiological method of insecticidal pests control is an alternative to chemical pesticides. Insect control agents are based on different microorganisms, which should be stably effective against target pest organism. There are different origins of industrial strains including isolation from natural objects, screening of collections, selection of existing strains, genetic engineering etc. but in all cases beneficial features of the strains should be preserved. In this article, the problems of preserving beneficial features of insecticidal bacteria *Bacillus thuringiensis* var. *israelensis* (BTH_{14}) are discussed. This strain is effective as the pest control agent against larvae of mosquitoes, midges and rice and champignons mosquitoes. Different methods and time of storage of various BTH_{14} strains are shown: 266/2 on meat-peptone agar (MPA) and in corpse of mosquitoes for one year, in sodium chloride crystals for one and a half year; 71 on MPA without reseeding for one year and for two years with reseeding every six months; 87a by cryopreservation for ten years; 87, 404, 19/43 as lyophilized bacteria for 28 years; 7-1/23, 71/82, 19/1 in sodium chloride crystals for 27 years. Culture of BTH_{14} strain was grown on MPA slants at 28-30 °C for 5-7 days until reaching the complete formation of spores and endotoxin crystals. Microscopic analysis was carried out with aniline black dye. Morphological analysis of colonies was performed with colony-purified BTH_{14}. When BtH_{14} was stored by the lyophilization method, the spore culture in a tube on a slant MPA was washed with 5 ml of a 20 % NaCl solution. Then 0.5 ml of the resulting suspension with a titer of 10^7-10^8 CFU/ml was added with a Pasteur pipette into glass ampoules, covered with a sterile swab, then sterile stopper and frozen in a cold bath at a temperature of –22 °C for 1 hour, dried at –45 °C for 23 hours, sealed under vacuum over a gas burner and stored in a refrigerator at 3-5 °C. When using the BtH_{14} storage method in NaCl crystals, 5 ml of 0.9 % saline was added to a tube with spore culture on slant MPA, resuspended, and 0.5 ml of suspension was added to sterile tubes, covered with cotton-gauze stoppers and stored at room temperature. When BtH_{14} was stored by cryopreservation, the spore culture of BtH_{14} was suspended in meat-peptone broth (MBP) with 10 % glycerol. The resulting suspensions (200 μl each) were poured into cryovials and stored at –80 °C. The BtH_{14} titer and larvicidal activity for *Aedes aegypti* mosquitoes were measured once or twice per year. The results showed that the culture of the 266/2 strain after a year of storage in the corpses of mosquitoes *Culex pipiens molestus* or on MPA dissociated with the formation of 0.8 and 1.6 % of the IV S form morphotype which lost activity against *A. aegypti* mosquitoes. The titer of the spores and the larvicidity of the 71 strain were at the initial level after one year of storage on MPA in tubes with paraffinized plugs when reseeded every 6 months. These indicators decreased, respectively, by 12 and 16 % in a year and by 25-27 % after 2 years of storage. Cryopreservation of the 87a strain provided stability of titer and larvicidal activity after 10 years. Thus, the initial titer and larvicidal activity expressed as LC_{50} for *A. aegypti* mosquitoes were 2.74×10^9 CFU/ml and 0.178×10^{-3} %, respectively. After 6 and 10 years, they corresponded to the following indicators: 2.82×10^9 CFU/ml and 0.19×10^{-3} %; 2.72×10^9 CFU/ml and 0.18×10^{-3} %. The BtH_{14} strains 7-1/23, 71/82, and 19/1 were stored in NaCl crystals. After 27 years of storage, their titers and LC_{50} for *A. aegypti* mosquitoes varied within the range of 3.12×10^9-3.52×10^9 CFU/ml and 0.135×10^{-3}-0.150×10^{-3} % as compared to the initial values that were

3.98×10^9-4.29×10^9 CFU/ml and 0.10×10^{-3}-0.11×10^{-3} %, respectively. The 87, 404, and 19/43 strains were stored by the method of freeze drying. After 28 years, their titers and larvicidal LC_{50} for *A. aegypti* mosquitoes remained within 3.32×10^9-3.68×10^9 CFU/ml and 0.11×10^{-3}-0.14×10^{-3} % as compared to the initial values 3.86×10^9-4.45×10^9 CFU/ml and 0.087×10^{-3}-0.103×10^{-3} %, respectively. Thus, the best indicators for preservation of valuable properties of BtH_{14} were obtained when stored in a lyophilized state, in NaCl crystals and using cryopreservation.

Keywords: *Bacillus thuringiensis* var. *israelensis* (BtH14), titer, storage, larvicidal activity

Pest management in vegetable, grains and fruit production present a serious economic problem, with the annual loss of national agricultural production reaches several billion rubles [1]. Biological preparations based on microorganisms of various origins are used to obtain environmentally friendly products, including bacteria, viruses, actinomycetes [1, 2], entomofluoric fungi and entomopathogenic nematodes [3, 4]. However, the review of international practice has indicated a preference for the use of entomopathogenic bacteria *Bacillus thuringiensis* (Bt) [5-7]. They have successfully been used as a safe entomocidal and growth-stimulating agent [8, 9]. In recent years, the number of *B. thuringiensis* varieties reported by domestic and foreign researchers has increased many times over and we now identify over 70 varieties [10]. *Thuringiensis* bacteria form spores, crystalline endotoxin [11-13], thermostable exotoxin [14], and sometimes enzymes with antifungal properties [15]. The advantages of Bt bacteria include their adaptability to streamlined production, wide spectrum of action [16-18], safety for humans and the environment [19, 20] and non-target insects [21-23]. To enable a cost-effective production of environmentally friendly biological products based on *B. thuringiensis* one should provide continuous supply of highly adaptable and virulent strain-producers, which requires the creation of collections and maintaining optimal terms and methods of storage.

It is customary to store bacterial cultures at the stage of their periodical transfer to fresh media. When applying this method, three basic conditions must be met: suitable maintenance medium, ideal storage temperature and transfer rate [24, 25]. Bt crop collections are stored on beveled meat-peptone agar (MPA), fish agar (FA) at a temperature of 3-5 °C in test tubes with unwaxed and waxed cotton-gauze plugs, under mineral oil, in insect corpses, in NaCl crystals, in lyophilized state and by cryopreservation.

Baktokulicid is a highly efficient, environmentally friendly biological product based on BtH_{14} for suppressing mosquitoes and midges (developed at the Russian Research Institute of Agricultural Microbiology in St. Petersburg, which has been tested in various ecological and geographical zones ranging from northern regions to the tropical belt (Russia, Belarus, Ukraine, France, Czechoslovakia, Cuba, India and Sri Lanka). Its activity is not inferior but very often is better than that of foreign analogues [26].

We apply different storage methods to study the integrity of technologically significant properties of BtH_{14} strains used in the production of Baktokulicid. In this paper, we for the first time invoked cryopreservation and used 20% NaCl as a protective medium for storing the lyophilized strains.

The purpose of the study is to assess the viability, productivity and larvicidal ability in a set of strains of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) when stored by various methods for varying lengths of time.

Techniques. The strain BtH_{14} 266/2 came from the CCEB collection (Culture Collection of Entomopathogenic Bacteria, Prague, Czechoslovakia). Strains 87, 404, 19/43, 7-1 / 23 and 71/82 were isolated from natural substrates (water, sludge and soil), and strains 71, 87a and 19/1 were obtained by the selection method. We studied the activity of BtH_{14} strains by applying different methods and storage periods: 266/2 — on meat-peptone agar (MPA) and in the

mosquitoe corpses *Culex pipiens molestus* 1 year, in NaCl crystals 1.5 years; 71 — on MPA 1 year without passage, 2 years with passages after 6 months; 87a — by cryopreservation for 10 years; 87, 404, 19/43 — in a lyophilized state for 28 years; 7-1/23, 71/82, 19/1 — in NaCl crystals for 27 years.

BtH_{14} culture was grown in solid nutrient MPA or FA media at a temperature of 29±1 °C until spores and crystals formed. When studying the morphological types of colonies, the culture was scattered in FA by using the depleting smear method. The microscopy was conducted by using black aniline dye [26] on day 7. Strain productivity was measured in yeast-polysaccharide media when grown by the in-depth method in Erlenmeyer flasks placed on a shaker with aeration (220 rpm) for 72 hours at 28 °C. The cell titer was measured by the conventional method of serial dilution with FA seeding.

Larvicidal activity was assessed by the WHO method [10, 28] on the *Aedes aegypti* larvae of instar IV. A suspension of the culture fluid (CF) was prepared by diluting in tap water 200, 400, 800, and 1600 thousand times, which corresponded to the conditional content of CF 0.5×10^{-3}; 0.25×10^{-3}; 0.125×10^{-3}; 0.0625×10^{-3} %, or 5.0; 2.5; 1.25; 0.625 μl CF/l. Portion of 50 ml of suspension was poured into Petri dishes at the appropriate dilution rate and 25 mosquito larvae were placed in there. Petri dishes were placed in a thermostat at 28-30 °C for 24 hours, after which the corpses were counted. The mortality rate for each concentration corrected for mortality under control was calculated using the formula:

$$X = (M_o - M_к)/(100 - M_к) \times 100\ \%,$$

where M_o and $M_к$ are the arithmetic mean values of the number of corpses in the test and control, respectively. The obtained data were used to calculate LC_{50}, expressed as the percentage of larvae killed by the Kerber formula [29]:

$$\lg LC_{50} = \lg C_M - \sigma\ (\sum X_2 - 0.5),$$

where C_M is maximum tested concentration of the preparation σ is the logarithm of the ratio for each previous dilution to the next dilution (logarithm of the multiplicity of dilutions) $\sum X_2$ is the sum of the ratio of the number of corpses to the total number exposed for the appropriate dilution.

Strains 87, 404 and 19/43 were lyophilized in 1988 by the following scheme. The culture was grown in the skewed MPA for 7 days, then the biomass was washed off with 5 ml of 20% NaCl, and a bacterial suspension with a titer of 10^7-10^8 CFU/ml was obtained. A portion of 0.5 ml suspension was transferred to glass tubes using a Pasteur pipette, and covered with a sterile cotton wool ball and then with a cotton plug. The interval between transferring the suspension to the tube and lyophilization was reduced to a minimum (max. 1 hour). The culture was frozen in a cold bath at a temperature of –22 °C for 1 h, then dried at –45 °C for 23 h after removing cotton plugs. The tubes were vacuum-sealed over a gas burner and put for storage in the fridge at 3-5 °C.

When BtH_{14} was stored in NaCl crystals, the culture was grown in MPA at a temperature of 30 °C for 5-7 days until the formation of spores and endotoxin. Physiological saline solution (5 ml) was introduced into the tube with the culture, then the biological material was carefully ground in a loop to obtain a homogeneous suspension, and 0.5 ml were added to the biological tubes, which were closed with ordinary cotton-gauze plugs and stored at 18-22 °C. Each sequence was repeated 20 times. We applied cryopreservation to freeze the culture at the stationary growth phase in 10% glycerol and placed it into the plate storage system (Liconic Instruments, Liechtenstein) at 80 °C [28]. To monitor BtH_{14} viability after freezing and assess baseline values of productivity and larvicidal activity, one of the replicates was de-frozen at 37 °C for 3 min and transferred to FA for further manipulations.

The obtained data were processed by the variance analysis method [29]

at 95% confidence interval. In the tables, the mean (*M*) and standard error of the mean (±SEM) are shown.

Results. The following changes in the morphological composition of the population of BtH_{14} 266/2 strain stored by three methods (Table 1) were observed. The colonies of four morphotypes were identified when disseminating to MPA: I-RS forms are grayish-white colonies with a slightly pink shade, rounded or irregularly rounded, flat, with a finely rough surface; spores and crystals of endotoxin were formed after 5 days. II-RS forms (pigmented) are lilac-pink colonies; no pigment was released into the medium. III-R forms are dull white, dry, wrinkled, flat, round colonies; the process of spore and crystal formation in agar media finished after 3 days. IV-S forms are creamy-beige colonies with blade-rugged edges; the chains of vegetative cells (deformed in many cases) were found in the 6-day culture placed in MPA. The ratio of colonies of various morphological types varied depending on the method of storage. I-RS phenotypes typical for the population ranged from 70.4 to 98.6%, whereas morphologically changed phenotypes ranged from 0.1 to 28.8% (see Table 1). The greatest variability was observed for the strain stored in the *C. pipiens molestus* larvae corpses. A total of 28.8% and 0.8% of III-R and IV-S colonies, respectively, were identified in addition to the dominant I-RS morphotype.

1. Natural variability of BtH_{14} 266/2 strain depending on the storage method (laboratory test))

Storage method and period	Viewed colonies	Colonies by morphotype, %			
		I-RS	II-RS (pigmented)	III-R	IV-S
MPA, 1 year	1147	98.3	0.1	–	1.6
NaCl crystals, 1.5 years	654	98.6	–	1.4	–
Culex pipiens molestus larvae corpses, 1 year	974	70.4	–	28.8	0.8

N o t e. MPA — meat-peptone agar. Dashes mean the absence of colonies of the corresponding morphological type. See the Results section for a description of morphotypes.

2. Biological activity of different morphotypes of BtH_{14} 266/2 strain depending on the storage method (*M*±SEM, laboratory test)

Morphotype	Storage method	Spore titre, $\times 10^9$/ml	LC_{50} for L4 *Aedes aegypti*, $\times 10^{-3}$ %
I-RS	MPA	2.55±0.10	0.21±0.04
II-RS (pigmented)		2.43±0.11	0.32±0.04
IV-S		1.23±0.12	0
I-RS	NaCl crystals, 1.5 years	3.23±0.11	0.19±0.04
III-R		2.20±0.12	0.26±0.04
I-RS	*Culex pipiens molestus* larvae corpses	2.28±0.09	0.24±0.04
II-R		1.21±0.12	0.38±0.04
IV-S		0.98±0.10	0

N o t e. See the Results section for a description of morphotypes.

The productivity in a liquid medium and larvicidal ability in relation to *A. aegypti* of different morphological variants was analyzed (Table 2). Colonies of morphotypes I and II in the culture stored in MPA had almost equal productivity: the titers reached $(2.55\pm0.10)\times10^9$ and $(2.43\pm0.11)\times10^9$ CFU/ml of culture liquid, respectively. In larvicidal activity reaching $(0.21\pm0.04)\times10^{-3}$ and $(0.32\pm0.04)\times10^{-3}$ % the morphotype I exceeded the morphotype II by 1.5 times. Morphotype VI culture grew slowly without spore and crystalline endotoxin formation and turned out to be non-pathogenic for *A. aegypti* larvae. The best results were shown by the method of BtH_{14} storage in NaCl crystals. The best results were shown by the method of storage in the corpses of mosquito larvae. Strain 266/2 was not stable and dissociated during storage with the formation of variants with reduced productivity, smooth S-variants lost 100% of their virulence. We managed to isolate by selection a more stable strain BtH_{14}

71, on the basis of which a method was developed for the preparation of a larvicide preparation.

Table 3 includes BtH_{14} strains' titers and activity data obtained by using different storage methods.

3. Biological characterization of 14 BtH_{14} strains after long-term storage (M±SEM, laboratory test)

Strain	Storage method and period	Spore titer, $\times 10^9$/ml		LC_{50} for L4 *Aedes aegypti*, $\times 10^3$ %	
		initial value	after storage	initial value	after storage
71	MPA, 1 year without passages	2.29±0.10	2.83±0.11	0.18±0.02	0.19±0.02
	MPA, 2 years with passsage every 6 months		2.19±0.10		0.21±0.02
87a	Cryopreservation:				
	immediately after freezing	2.74±0.12		0.178±0.02	
	after 3 years		2.78±0.11		0.18±0.02
	after 6 years		2.82±0.10		0.19±0.02
	after 10 years		2.73±0.10		0.19±0.02
87	Lyophilization, 28 years	4.45±0.14	3.68±0.12	0.087±0.02	0.11±0.02
404	Lyophilization, 28 years	4.34±0.12	3.42±0.10	0.092±0.02	0.12±0.02
19/43	Lyophilization, 28 years	3.86±0.11	3.32±0.12	0.103±0.02	0.14±0.02
7-1/23	NaCl crystals, 27 years	4.29±0.11	3.52±0.12	0.10±0.02	0.135±0.02
71/82	NaCl crystals, 27 years	4.18±0.14	3.28±0.13	0.11±0.02	0.142±0.02
19/1	NaCl crystals, 27 years	3.98±0.12	3.12±0.12	0.108±0.02	0.15±0.02

N o t e. MPA — meat-peptone agar..

Comparison of titers and larvicidal activity in different strains of BtH_{14} depending on the method of storage showed (Table 3) that the strain 71 managed to preserve almost all its properties after 1 year storage in MPA with waxed cork. The strains passaged every 6 months lost their properties by 12 and 16% after one year, and by 25 and 27% after two years. The cryopreserved strain 87a managed to preserve its high adaptability to streamlined production and larvicide ability after 10 years of storage. Strains 7-1/23, 71/82 and 19/1 continued to have high titers and larvicidal activity after storage in NaCl crystals for 27 years. The same must be said for the lyophilized strains 87, 404 and 19/43, which were stored in tubes for 28 years. After the lyophilized and cryopreserved BtH_{14} strains have been sown in FA using the depleting smear method for long-term storage, no significant variability was obtained.

The occurrence of atypical forms in the course of storage is quite natural. On the basis of the facts obtained, we conclude that neither cryopreservation, nor lyophilization, nor NaCl crystals should be considered as a basis for contrasting different methods of BtH_{14} storage, on the contrary, the methods should be used complementary to each other, with support of tests for viability and purity, productivity assessment and biotesting prior to the season in which the bacterial larvicidal preparation is to be tried and tested [1].

The stock of Bt strains is constantly replenished. The strains isolated from natural substrates are firstly assessed for entomocidal activity and productivity. The selected active strains are deposited and put into the plate storage system for long-term and loss-free storage of cultures, as well as for accurate labeling, registration and tracking of samples. RCAM database (Russian Collection of Agricultural Microorganisms), which is available on-line (http://62.152.67.70/cryobank/login.jsp), supports about 40 strains of *Bacillus thuringiensis* of various serotypes (depending on the effect on insect pests): var. *thuringiensis* (BtH_1), var. *darmstadiensis* (BtH_{10}), var. *israelensis* (BtH_{14}), var. *kurstaki* (BtH_{3a3b}) [30].

So, as can be seen from the above, the strains of *Bacillus thuringiensis* BtH_{14} can be efficiently stored with the use of cryopreservation, lyophilization and NaCl crystals. Analysis of commercial producers with larvicidal effect for viability, purity, productivity and activity should be performed prior to the sea-

son in which bacterial larvicidal preparations are to be tried and tested at biological factories and laboratories. Earlier studies have shown that the same storage methods could be used for storing *B. thuringiensis* cultures of other serotypes.

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MATERIALS DERIVED FROM *Amaranthus cruentus* L. USED AS CO-SUBSTRATES CAN INTENSIFY METHANOGENESIS DURING BIOCONVERSION OF ORGANIC WASTE

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Abstract

Methane fermentation (biomethanogenesis) performed by a multicomponent microbial consortium under anaerobic conditions results in a mixture of approximately 65 % CH_4, 30 % CO_2, 1 % H_2S and minor amounts of N_2, O_2, H_2 and CO. The peculiarity of biomethanogenesis lies in the ability to convert almost all classes of organic compounds, household, agricultural and some industrial waste into biogas. We were the first to assess the efficiency of the biogas production from organic waste as influenced by various materials derived from amaranth (*Amaranthus cruentus* L.) which were used as co-substrates. Our findings indicate that optimization of the substrate organic matter composition by using dry phytomass of amaranth plants or amaranth pulp which remains after removing all practically valuable substances makes it possible to produce biogas from sewage sludge. This facilitates solving ecological problems of waste disinfection and utilization, and gives us an alternative, cheap and renewable source for fuel. Cultivated *A. cruentus* is a high-yielding protein-rich crop. Its biomass serves as a reproducible raw material. In our previous works, we reported the technology for rutin, vegetable protein and pectin production from *A. cruentus* plants, and suggested a scheme for complex processing which includes extraction of these substances from amaranth dry phytomass in a single technological cycle. The pulp obtained after extraction of all valuable compounds was proposed as a co-substrate for organic waste anaerobic fermentation. We modeled the effect of amaranth-derived substances on biogas production in the laboratory bioreactor using large-tonnage urban sewage sludge as a substrate. It was shown that the doses of the additives affected the process, i.e. the excess of amaranth plant mass (74 % and 87 %) suppressed methanogenesis. The thermophilic (50 °C) fermentation was found to be superior to the mesophilic one (37 °C), with the biogas production of 354 ml per gram of dry matter, when large-tonnage sewage sludge after filter press (45 % humidity) was fermented using amaranth pulp as the co-substrate. Moreover, in the presence of amaranth pulp, the biomethanogenesis under the mesophilic conditions also increased, the lag phase was almost absent, and the CH_4 level throughout the experiment was about 60 %. As a result, the specific biogas yield reached 251.9 ml per gram of dry matter that is equivalent to ~ 0.25 m^3 of the resultant biogas from 1 kg of organic raw material dry matter. In order to search for the active fraction of amaranth phytomass, we used solvents of different polarity, i.e. dichloromethane, 70 % aqueous ethanol and distilled water. It was found that the lag phase reduced to 10 days with the CH_2Cl_2 and EtOH extracts, which was comparable to that in the presence of dry amaranth phytomass. Obviously, these extracts contain components which either undergo rapid destruction by microorganisms able to turn them into biogas, or contribute to bacterial growth. The dichloromethane extract added to the substrate led to the most efficient biogas production, which is consistent with

the literature data. Our findings indicate the ecological and economic feasibility of using amaranth pulp for organic waste bioconversion.

Keywords: *Amaranthus cruentus* L., amaranth, methanogenesis, co-substrate, amaranth phytomass extracts, biogas, sewage sludge, amaranth pulp

Methanic fermentation, or biomethanogenesis, is a known process of biomass-to-energy conversion [1]. Biogas (a mixture of approximately 65% CH_4, 30% CO_2, 1% H_2S and minor amounts of N_2, O_2, H_2, CO) is formed in anaerobic environment, by multielement microbial consortium, capable of converting almost all classes of organic compounds, household, agricultural and some industrial waste into biogas [2]. Methanogenesis is executed by utterly specialised prokaryotes, being very old archebacteria, or archea from the *Methanobacterium*, *Methanosaeta* (*Methanothrix*), *Methanococcus*, *Methanosarcina*, *Methanocorpusculum*, *Methanobrevibacteria* and *Methanopyrus* genera [3-6].

The review of existing works related to obtaining of biogas [7-10] has proved the problem on intensifying of methane fermentation of organic raw material is still urgent. One of the most promising trends consists in search for plant simulators and inhibitors of methanogenesis. E.g., a comparison was made between the composition of volatile fatty acids (VFA) and the output of biogas under the effect of 13 plant extracts, selected according to the highest flavonoid activity [11] (by fermentation of 50:50 mixture of herbal phytomass extract and barleycorn). Experiments found that extracts of *Lavandula officinalis* and *Solidago virgaurea* stimulate fermentation, while those of *Equisetum arvense* and *Salvia officinalis* inhibit the yield of methane. Of special interest is the work that deals with investigation into effect the herbaceous plant extracts (21 species) have on methanogenesis, gram-positive and gram-negative bacteria, their antimicrobial potential, and destruction of dry substance in vitro [12]. The extracts were obtained through the use of methanol, acetone or water, and the content of total sugars, tannins and saponines was determined. Antimicrobial potential was estimated at gram-positive streptococci and staphylococci and gram-negative bacteria *Escherichia coli* and *Enterobacter*. Acetone and methanol extracts of *Eucalyptus globulus* and water extract of *Sapindus mukorossi* and *E. globulus* inhibit methanogenesis in vitro. The study the effect that saponine-rich extracts of *Carduus, Sesbania* and *Knautia* leaves, and of common fenugreek seeds (*Trigonella foenum-graecum*) have on fermentation in the scar, the output of methane and the microbial community [13] has proved that saponines have antiprotozoal activity which is why they do not suppress methanogenesis. The saponines of common fenugreek seeds boost the activity of the scar content and affect the microbial community by reinforcing growth of fiber decayers and suppressing the growing population of fungi.

Various plant biomass added in sediments of treatment facilities has gained wide-spread acceptance in increasing of methane tanks capacity [14]. Added co-substrates, e.g. manure, enrich carbon-poor substrate with an organic substance. Europe employs plants in producing of more than 50% of its biogas [15, 16]. Still urgent is the search of co-substrates for effective bioconversion of organic waste from agriculture and municipal waters into high-methane biogas.

We were the first in studying phytomass and pulp of cultivated amaranth, obtained after extraction of all practically valuable substances, as co-substrates, and in finding of its optimum proportion with substrate (sediment of municipal waters) to enhance formation of biogas and methane fermentation.

The work assessed the effect the red amaranth-based additives have on effective conversion of sedimentary sludge in treatment facilities and of organic waste into high-methane biogas.

Techniques. As a substrate, we used sediments of municipal waters (SMW, humidity 80.4%; compacted SMW, humidity 98.4%; SMW after press-

filter, humidity 45.0%) of the city of Kazan, as co-substrates, phytomass, pulp after comprehensive processing (extraction of pectines, rutin and plant protein), and alcohol, dichlormethane and water extracts of pulp of amaranth (*Amaranthus cruentus* L.) plants (Dyuimovochka variety). To find elemental composition (C, H, N and S) of substrate and co-substrate, we employed an analyzer CHN-3 (Khimavtomatika, Russia); streptocide was a standard (C — 41.85 %, H — 4.65 %, N — 16.26 %, S — 18.58 %).

Experiments varied type of substrate, type of co-substrate, substrate-co-substrate ratio, temperature of incubation. Fermentation was made in laboratory reactors that consists of water bath LB-160 and immersion circulating thermostat LT-100 (bottles made of МТО БК3-50 glass, V = 500 ml) (LOIP, Russia).

To compare methane formation depending on co-substrate fraction mixed with SMW (24, 52, 74 and 87 % on absolute dry weight, calculated with regard to humidity of SMW and co-substrate), we used dried phytomass of amaranth; to all sample we added 100.5 g of compacted SMW and fermented at 37 °C. The effect of mesophilic (37 °C) and thermophilic (50 °C) modes of incubation was researched during fermentation of SMW after the filter-press (50.0 g substrate with 100 ml of distilled water added). The same experiment studied the effect the amaranth pulp, as a co-substrate, has on effectiveness of mesophilic (37 °C) fermentation (22.5 g substrate, 16.7 g pulp with 9.2 % humidity; 100 ml of distilled water). To understand the effect of pulp extracts, 0.2 g of dichloromethane (CH_2Cl_2), 0.2 g of alcohol (EtOH) or 2.3.g of amaranth water extract (obtained by evaporation to dryness in rotary evaporator IR-1LT, Russia) was added to substrate (mixture of 37.5 g of SMW and 105.0 g of compacted SMW); for comparison, extracts were replaced with amaranth pulp (5.0 g) (incubation at 37 °C).

Daily volume measurements estimated the output of biogas [17]. Gas-liquid chromatography (GLC) controlled the methane content in samples (CHROM-5, Laboratorní přístroje, Czechia, column 2.4 m with Porapak Q filler, 80-100 mesh, Sigma-Aldrich Co., USA; 80 °C; heat conduction detector, carrier gas helium).

Changes in microorganisms composition in the process of SMW fermentation have been revealed by culture on methanogenic media [18] and Gram staining with further microscopy (MBI-15, LOMO, Russia).

Origin 6.1 siftware was used to process data (https://softadvice.informer.com/Origin_6.1_Free_Download.html). Tables and figures show means (*M*) and standard errors of means (±SEM). Significance of differences was estimated by Student's *t*-criterion. The distinctions were considered statistically significant at $p = 0.05$.

Results. Amaranthus cruentus was chosen because protein-high biomass [19-21] of this high-yielding crop can be an industrially reproducible plant raw material. As a part or research, we developed unique methods and scheme for obtaining rutin, plant protein and pectin during comprehensive processing of amaranth [20] based on extraction from dried phytomass in a single technological cycle.

1. Elemental composition of sediment of municipal waters (SMW), phytomass and amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) pulp used for laboratory simulation of the biogas production

Substrate, co-substrate	C, %	H, %	N, %	C/N
Compacted SMW	34.65	6.20	7.15	4.9
SMW	39.82	7.05	5.81	6.9
Amaranth phytomass	36.98	4.67	3.47	10.7
Amaranth pulp	42.11	6.42	5.20	8.1

Tables 1 and 2 contain properties of substrates and co-substrates used in the experiment. Methanogenesis was simulated in laboratory, with the use of bioreactor (Fig. 1).

2. Properties of substrates having various content of dried amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) phytomass used for laboratory simulation of the biogas production (n = 3, M±SEM)

Amaranth, %	Dry substance, g	Organic dry substance, g	Humidity, %
24	11.2±0.34	7.3±0.22	92.1±2.76
52	15.9±0.48	11.3±0.34	89.0±2.67
74	18.1±0.54	13.7±0.41	86.7±2.60
87	21.5±0.65	16.9±0.51	83.2±2.50

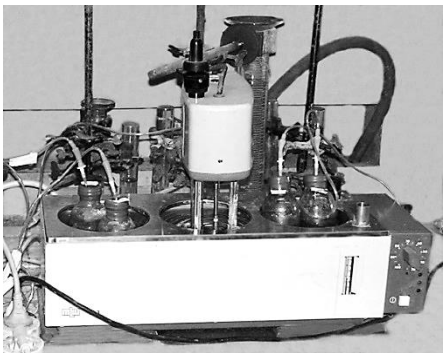

Fig. 1. Laboratory facility for biogas obtaining (V = 500 ml, LOIP, Russia).

Kinetics of CH_4 formation with 24, 52, 74 and 87 % of dried amaranth phytomass added as co-substrate (Fig. 2) has proved the biogas yield is the highest at 24% (291.1 ml/g of dry substance, p = 0.05). The effect retained for 50 days (Table 3), with approximately 60% of CH_4 in biogas (see Fig. 2). More objective criterion of the process efficiency is a specific yield of biogas in terms of content of organic dry substance in the substrate that, too, was high (see Table 3).

Adding of 52% of amaranth produced 226.6 ml/g of biogas in dry substance (true at p = 0.05). Biogas had been forming for 138 days and contained, approximately, 55% of methane (see Fig. 2).

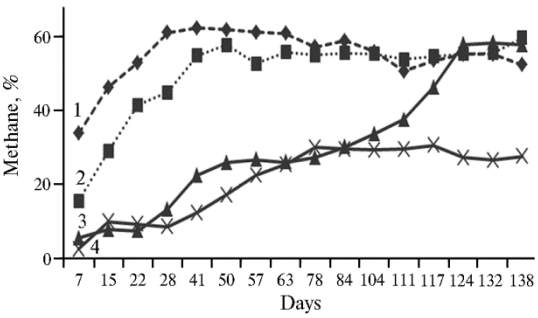

Fig. 2. Kinetics of CH_4 formation during bioconversion of sediment of municipal waters vs. content of amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) dry biomass in the substrate: 1 — 24 %, 2 — 52 %, 3 — 74 %, 4 — 87 % (average for 3 repetitions, laboratory trial).

With 74% amaranth phytomass content, the process had prolonged lag phase: gas formation activated no sooner than in 110 days. Excess of amaranth suppressed methanogenesis, and specific output of biogas made 127.8 ml/g in dry substance (see Table 3). With 87% amaranth, biogas contained not more than 30% of methane (see Fig. 2) at biogas yield of 29.1 ml/g in dry substance (p = 0.05, see Table 3).

3. Production of biogas during bioconversion of sediment of municipal waters vs. content of amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) dry phytomass in substrate (n = 3, M±SEM, true at 5% significance level, laboratory trial)

Amaranth, %	Specific yield		
	ml gas/ml substrate	ml gas/gdry substance	ml gas/g organic dry substance
24	23.0±1.15[a]	291.1±14.60	445.1±22.26
52	24.9±1.25[a]	226.6±11.33	318.5±15.93
74	17.0±0.85	127.8±6.39	168.4±8.42
87	4.7±0.24	29.1±1.46	36.9±1.85

N o t e. Between variants marked as [a] there are no statistically significant differences at p = 0.05.

A comparison of these results with the data presented in the Table 2 has

proved optimum humidity of substrate for biogas should be not less than 90%, and gas formation decreases with its reduction. Consequently, adding of amaranth phytomass to substrate was only effective at certain quantitative relationship with SMW, whereas excessive amaranth phytomass suppressed methanogenesis.

Figure 3 illustrates dynamics of SMW pH change with 52% amaranth phytomass added.

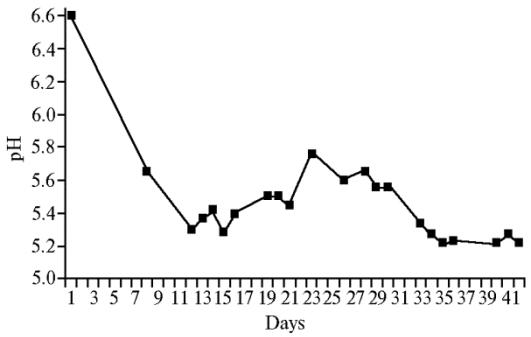

Fig. 3. Rise of acidity during anaerobic fermentation of sediment of municipal waters with 52% dry biomass of amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) (average for 3 repetitions, laboratory trial).

Lag phase in mesophilic mode took 30 days, when co-substrate was amaranth pulp, remained after comprehensive processing and extraction of pectines, rutin and plant protein [22, 23]. On obtaining of maximum daily yield of biogas (appr. 120 ml on day the 40), high content (60%) of CH_4 was noted in it (Fig. 4). Specific productivity in experiment made 134.7 ml/g of dry substance ($p = 0.05$, Table 4). In thermophilic mode, the volume of the gas formed was considerably greater: for longer than 20 days, its yield exceeded 200 ml. In addition, lag phase reduced to 14 days when biogas contained more than 50% of CH_4 (see Fig. 4).

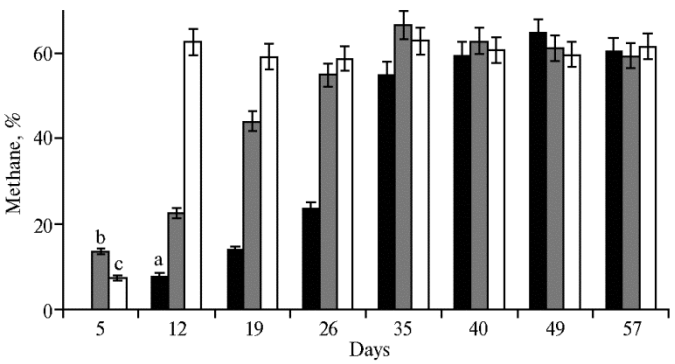

Fig. 4. Content of CH_4 in biogas at various temperature modes for anaerobic fermentation of sediment of municipal waters (SMW) **in the presence of amaranth (*Amaranthus cruentus* L., Dyuimovocka variety) pulp:** a — SMW at mesophilic mode (37 °C), b — SMW at thermophilic mode (50 °C), c — SMW, and pulp at mesophilic mode ($n = 3$, $p = 0.05$, laboratory trial).

4. Biogas production during bioconversion of sediment of municipal waters (SMW) **vs. temperature mode and content of amaranth (*Amaranthus cruentus* L., Dyuimovochka variety) pulp in substrate** ($n = 3$, $M \pm$SEM, true at 5% significance level, laboratory trial)

Composition	Mode	Specific yield	
		ml gas/ml substrate	ml gas/g dry substance)
SMW	Mesophilic, 37 °C	20.5±1.03[a]	134.7±6.74
SMW + amaranth pulp	Mesophilic, 37 °C	22.7±1.14[a]	251.9±12.60
SMW	Thermophilic, 50 °C	53.1±2.66	354.0±17.70

N o t e. Between variants marked as [a] there are no statistically significant differences at $p = 0.05$.

Specific yield of biogas made 354.0 ml/g of dry substance ($p = 0.05$), that points to the advantage of thermophilic mode of SMW amaranth-free fermentation. Amaranth added to substrate increased yield of biogas in mesophilic mode (with lag phase almost omitted, on day 12 the volume of biogas reached 80 ml for 62% of CH_4) (see Fig. 4). It was only after 60 days, that yield of gas dropped to 20 ml, with approximately 60% of CH_4, before the experiment had been completed. With amaranth pulp added in mesophilic mode, specific yield of biogas made ~ 0.25 m^3 per 1 kg of dry substance of organic raw material.

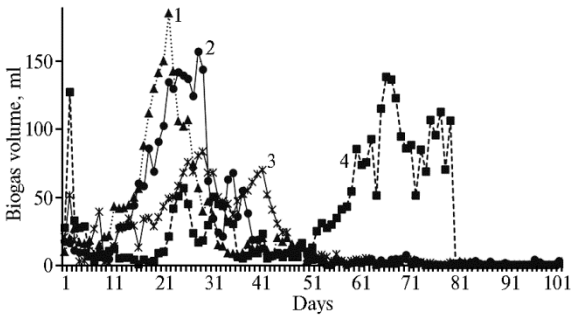

Fig. 5. Kinetics of biogas production during bioconversion of sediment of municipal waters with added alcohol (1)**, dichlormethane** (2) **and water** (3) **extracts and amaranth (*Amaranthus cruentus* L., Dyuimovocka variety) pulp** (4) (average for 3 repetitions, laboratory trial).

Dichlormethane extract of amaranth phytomass [22] has green color through the presence of chlorophyll, water extract contains carbohydrates, proteins, mineral salts and amarantite, whereas the ethanol extract is rich in phenol compounds rutin and quercetin. After three extractions, the pulp contained fiber, carbohydrates and pectines [22]. Kinetics of gas formation observed during mesophilic fermentation of SMW and compacted SMW mixture (Fig. 5) has proved that CH_2Cl_2 and EtOH extracts added to substrate reduced lag phase to 10 days. This effect is comparable with that of amaranth phytomass. Clearly, dichlormethane and ethanol extracts contain components that either destruct easily when exposed to microorganism community converting them to biogas, or help in the growth of biomass. CH_2Cl_2 extract added to substrate marked the most effective formation of biogas, which is consistent with the literature data [11, 24-27] (Table 5).

5. Production of biogas during bioconversion of sediment of municipal waters with various extracts and amaranth pulp (*Amaranthus cruentus* L., Dyuimovochka variety) added (n = 3, M±SEM, true at 5% significance level, laboratory trial)

Co-substrate	Specific yield		
	ml gas/ml substrate	ml gas/g dry substance	ml gas/g organic dry substance
CH_2Cl_2 extract	162±0.81[a]	266.1±13.31[b]	433.5±21.68
EtOH extract	14.5±0.73[a]	236.1±11.81[c, d]	382.5±19.13[e]
Water extract	16.6±0.83[a]	221.8±11.09[c]	345.8±17.29[e]
Amaranth pulp	23.7±1.19	259.1±12.96[b, d]	355.4±17.77[e]

N o t e. Between variants marked as [a] there are no statistically significant differences at p = 0.05.

With pulp added, specific yield of biogas made 259.1 ml/g of dry substance (p = 0,05), that can be related to a similar result (266.1 ml/g of dry substance) for extract obtained through the use of CH_2Cl_2, with 82.5% of CH_4 on day 98 (maximum value over the whole period of studies). We point out that both amaranth pulp after three extractions (see Table 5) and amaranth phytomass (see Table 3) serve as an activating co-substrate for methanogenesis (RF Patent No. № 2351552). Hence, in the presence of amaranth pulp, biomethanogenesis, too, increases its productivity in mesophilic mode (by 12.8 %, p = 0.05), that, in general, increases the efficiency of comprehensive processing of raw obtained during growing of this plant. The results presented have confirmed environmental and economic feasibility of amaranth pulp use.

Microbiological study of samples during anaerobic fermentation of SMW has proved that typical of the original substrate is the presence of large eukaryotic forms. Gram-positive small rod bacteria, both single ones and in long chains, dominated in the medium at the maximum activity of gas formation (day 40). Very small single forms were more typical for acetate-based nutrient medium. Smooth and rough colonies grew on MPA [18]. Considerable amount of very small colonies has grown at the medium for methanogens. Both gram-positive and gram-negative forms were found.

Eventually, our studies have proved that optimized substrate of organic

substance with the use of amaranth phytomass/pulp affords higher effective production of biogas from the sediment of municipal waters, thus attacking the problem of waste disposal and getting the fuel from a cheap renewable source.

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