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Reviews, challenges

ANTHRAX: LIFE CYCLE, MECHANISMS OF PATHOGENESIS AND PROSPECTS IN THE DEVELOPMENT OF VETERINARY VACCINES

(review)

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Abstract

Anthrax is an acute *especially* dangerous disease of agricultural and wild animals, as well as humans. Anthrax is induced by the gram-positive spore-forming bacterium *Bacillus anthracis*. This infection is global, but the incidence rate of livestock and people varies depending on the environmental situation and the implementation of control strategies (C.J. Carlson et al., 2019). Historical and modern experience suggests that uncontrolled outbreaks of anthrax can have disastrous consequences. This review describes the life cycle of the pathogen, environmental features of the anthrax spread and mechanisms of pathogenesis. Given these factors we discuss the optimal strategies that have been developed over the years taking into account the cost and outcome for combating the dangerous infection. The timely disposal of dead animals and the vaccination of healthy livestock, used together, can effectively stop the spread of the disease. Thus, the development of highly effective, safe and low-cost vaccines is extremely relevant and, moreover, in fact the only promising method for improving the epizootic situation with this hazardous disease. Vaccination of farm animals for several decades has significantly reduced the risk of anthrax, but it is not mandatory in many countries and is often used only after onset of the disease, and not to prevent it. Despite a significant decrease in the incidence rate, the current situation with anthrax in the Russian Federation is characterized as unstable (A.G. Ryazanova et al., 2018; E.G. Simonova et al., 2018). Animal epizootics and human cases are still being recorded in the country due to the presence of natural soil reservoirs of the pathogen and incomplete coverage of vaccination for farm animals. Currently, only live attenuated vaccines are used to vaccinate animals. The review summarizes their effectiveness and safety, as well as the limitations associated with the use of attenuated vaccines. Although existing vaccines have been shown to be effective, they have several serious flaws. Certainly, the relevance of the development of more effective veterinary vaccines against anthrax, based on modern approaches, is fully justified. In particular, there is a need to design a veterinary vaccine that does not contain the pathogen in any form and is compatible with the use of antibiotics, which are necessary, both during the outbreak of anthrax and for regular use in the treatment of various animal diseases. The application of new approaches, the devising modern recombinant vaccines and the rejection of the use of pathogens in an attenuated form is an important and promising task. This review provides an analysis of studies on the development of new candidate vaccines against anthrax. The main attention is paid to the development of subunit vaccines using *B. anthracis* recombinant antigens obtained in various expression systems, including vaccines for oral administration and compatible with antibiotics.

Keywords: anthrax, *Bacillus anthracis*, veterinary vaccines, recombinant antigens

Anthrax is an extremely dangerous zoonose disease caused by a gram-positive spore-forming bacterium *Bacillus anthracis* (pathogenicity group 2).

Nearly all mammals, including humans, are susceptible to anthrax [1]. Soil containing *B. anthracis* spores is a source of infection for herbivores. High resistance of anthrax spores to the environmental factors, their ability to persist in the soil for a long time and to turn into a vegetative form under certain conditions makes the fight against this infection an extremely difficult task of medicine and veterinary. Humans get infected by direct contact with contaminated objects of animal origin. From 2000 to 20000 human anthrax cases are reported annually [2]. It is considered that 1.83 billion people live in the areas with the risk of anthrax outbreak [3]. In some regions disease outbreaks are a consistent and predictable ecosystem peculiarity and take their place at the certain stage of the seasonal cycle, in other areas epizootics are rare and are capable to cause massive livestock and wild animals deaths [4-7]. Climatic conditions are considered to be a generally accepted factor of the activation of natural foci and a risk factor of anthrax epizootics among herbivores, especially in endemic regions [8-12]. Despite the fact that anthrax has been being studied for about 130 years, there are many unresolved issues related to the pathogenesis and epidemiology of the disease [13].

Nowadays modern veterinary vaccine invented in 1937 [14] is used in most countries. This vaccine is a non-encapsulated toxigenic Sterne 34F2 strain or its analogues (the Russian Federation, China, Romania). Although existing vaccines have been shown to be effective, they have several disadvantages. In particular, the parenteral route of vaccine administration is impractical for mass immunization of animals, especially for animals on free grazing. However, peroral administration of Sterne 34F2 vaccine is turned out to be ineffective [15]. The design of new concepts and the rejection of attenuated pathogens as a vaccine basis are important and prospective research tasks.

This review discusses the relevance of new-generation recombinant anthrax vaccines and modern methods and approaches used for their development. The effectiveness and safety of live attenuated vaccines use is also discussed in current paper.

Lifecycle of the causative agent, ecological and epizootic features of anthrax. Anthrax is a natural-focal sapronous disease [16, 17]. The disease mainly affects herbivores. Cattle, horses, deer, sheep and goats are the most susceptible to *B. anthracis* animals. In certain cases anthrax epizootics taking place in the wild can lead to infection of farm animals and humans. *B. anthracis* occurs in two forms – as vegetative cells and as spores. This fact determines crucial anthrax epizootic features. It is generally accepted, that alimentary infection rout is the main one. In alimentary rout spores enter the animal's organism with food or water. However, the infection through the skin and respiratory tract is possible as well. At the same time, anomalies that often occur in anthrax epizooties are difficult to explain by the direct transition of spores into organism during cattle grazing. Apparently, insects are able to be spore carriers in unpredictable disease outbreaks [18-20]. Human's activities and ecological factors can also play an important role in the occurrence of anthax epizootics [12, 21-23]. After infection and death of animals *B. anthracis* vegetative cells enter the environment (with blood and other biological fluids) and turn into spores when exposed to oxygen. Seeding the soil with spores after the death of sick animals is obligatory for the continuity of anthrax causative agent circulation. *B. anthracis* spores can be translocated from primary soil foci to other areas (including ones that previously were free from the infection) with the participation of scavengers or insects (biotic processes) or due to abiotic factors such as water or wind [19, 20, 22, 24]. Spores are extremely resistant to environmental factors and can persist in the soil for decades [23]. An outbreak of anthrax in deer in the Yamalo-Nenets Autonomous Okrug in 2016 (75 years after the last known case in the region) indicates that the

potential duration of *B. anthracis* persistence in the environment may exceed centuries [11]. However, the quantity of spores in soil and water samples in the areas of burials of animals that died because of anthrax rapidly decreases to a level that is below the detection threshold, which can be explained by an effective dispersion of spores by animals, rainwater and wind alongside with a relatively low viability of *B. anthracis* vegetative cells and spores in the environment [8]. It was hypothesized that *B. anthracis* reproduces in the soil in so-named incubation zones before the contamination of animals. Calcium and organics-rich soils at pH > 6.0 and temperature above 15 °C favor the bacteria reproduction in topographically well-defined areas in which the infection of animals and anthrax outbreaks occur [8, 10]. Under the modeled environmental conditions it has been shown that the spores of the virulent Ames strain and vaccine Sterne strain were able to germinate and to reproduce intracellularly in a free-living soil amoeba (*Acanthamoeba castellanii*). The 50-fold increase in the number of spores was observed after 72 hours of inoculation [25]. In another work it was demonstrated that inoculation with the spores of rhizosphere can also lead to their germination [26].

It turned out that bacteriophages can play an important role in the anthrax lifecycle as well. Lysogeny may stimulate or, conversely, block sporulation (depending on the bacteriophage) inducing phenotypic changes in *B. anthracis* and providing a long-term bacterial colonization both in the artificial soil environment and in the manure worm (*Eisenia fetida*) intestines [27]. It has been shown that vegetative cells can persist in the soil for up to 120 hours, and a mixture of vegetative cells and spores inserted into the upper soil layer remains in it for 56 days [28]. The constant host-soil-host cycle is a basis for recurrent outbreaks. People are infected with anthrax through the contact with infected animals, and several cases of the disease transmission through insect bites were described as well [1]. The possibility of transmission of anthrax from a sick human to a healthy one has not been documented. Any anthrax outbreak can be disastrous if left uncontrolled. Anthrax usually develops very quickly in most animals and the successful treatment is not always possible. The disease is characterized with a global spread, but the incidence of anthrax in the farm animals and humans varies depending on local ecology and the implemented disease control strategies [3]. The decades of vaccination of farm animals in economically developed countries have reduced the risk of anthrax significantly. The refusal to vaccinate reindeer over the previous 9 years was the main reason for anthrax epizootic in the Yamalo-Nenets Autonomous Okrug in 2016, when over 2600 reindeer died and 36 people fell sick (with one lethal outcome) [11]. Most developed countries report sporadic anthrax cases in livestock and humans, but this disease still remains enzootic in some regions of Africa, in the Middle East and in Central Asia. Despite the significant decrease in incidence, current anthrax situation in the Russian Federation is still characterized as unstable [29-31]. Anthrax epizootics and human anthrax cases are still documented in Russia. This is due to the presence of natural soil reservoirs of the pathogen and incomplete vaccination of farm animals coverage. In the Russian Federation there are over 35 000 stationary unfavorable anthrax points (SUP). At the same time, there is a high probability of the presence of a significant number of unaccounted burials [32]. Most of anthrax outbreaks documented in recent years have been occurred in accounted SUPs, and in some of them the activity of the anthrax soil foci has not been manifested for the last 40-60 years. At the same time, some epizootic foci have appeared in the territories considered to be safe [33]. There are also reports of anthrax outbreaks in the wild in various ecosystems all over the world [5, 7, 34].

It is a mistake to treat anthrax a legacy of some sanitary troubles of the past like improperly utilized livestock buries etc. Apparently, there are multiple

natural reservoirs of the pathogen, which has phases of rest and phases of active development in its lifecycle. Spores can be activated due to natural and climatic processes: floods or droughts, dramatic shift in temperature, thawing of the soil, landslides etc. In particular, it can be confirmed by the periodic mass deaths of wild animals from anthrax in the protected wild areas in Africa. For instance, hundreds of hippos died from anthrax in 2017 in Bvabvata National Park in northeastern Namibia during an extremely low water level in rivers [7]. Yamal anthrax outbreak is also associated with an unusual heat, which contributed to an increase in seasonal thawing of permafrost to 1 m and the movement of spores from deep layers to the soil surface. Such soil conditions are favorable for the sporulation and vegetation of the pathogen. Climatic factors additionally contributed to an increase in the number of the blood-sucking insects, which are considered as carries and a reason for the rapid spread of epizootics [11, 16, 36). The activity of emergent focus is only manifested in the disease and death of animals. At the same time, natural foci themselves often cannot be detected and localized. Common practice for controlling infection in cattle includes the utilization of animal carcasses and vaccination of grazing animals.

The search for optimal strategy to combat the spread of the disease both in terms of cost and effectiveness has recently been performed using modern methods of mathematical modeling [36]. The results of American researchers' calculations indicate that only the combination of timely disposal of animal carcasses with vaccination of healthy livestock is able to stop the spread of anthrax. This makes the development of modern cheap and effective anthrax vaccines extremely relevant and, moreover, the only prospective way to improve the epizootic situation with such a dangerous disease as anthrax.

Pathogenesis mechanisms. Once *B. anthracis* spores enter the organism of a susceptible host (via gastrointestinal or inhalation rout or through the skin) they can locally germinate into a vegetative form at primary contact sites. Also, spores can be captured by macrophages and be translocated into the lymph nodes, where they germinate, migrate into the bloodstream and release the system effects-causing toxins. The main virulence factors of the vegetative form of *B. anthracis* are a capsule of poly-γ-D-glutamic acid and anthrax toxins. The capsule has low immunogenicity and makes the bacterium resistant to phagocytosis and the complement system. Capsule components are encoded by the pXO2 plasmid. As a result, pathogen becomes invulnerable to the host's immune system [37, 38]. Anthrax toxins are three proteins encoded by pXO1 plasmid – protective antigen (PA), lethal factor (LF) and edema factor (EF), which combine into binary complexed PA/LF and PA/LF to form a lethal toxin (LT) and edema toxin (ET), respectively [39].

PA is an 83 kDa protein (PA83). After PA83 binds to cellular receptors (capillary morphogenesis protein 2 — CMG2 and tumor endothelial marker 8 — TEM8), it undergoes proteolytic hydrolysis by cellular surface furin-like proteases. As a result of the proteolysis, a non-engaged in the receptor binding N-terminal 20 kDa PA fragment is being split off leaving only the 63 kDa C-terminal PA fragment bound to the receptor. CMG2 receptor has a higher affinity to PA83, is widely expressed in various types of cells and is considered to be the main anthrax toxin receptor mediating *in vivo* lethality, while TEM8 plays a minor role in the pathogenesis of anthrax [40]. Two anthrax receptors have a high degree of identity with each other and contain a conservative domain that binds PA [41, 42]. CMG2 is highly conservative for various animal species (for example the degree of identity of human and mouse CMG2 is 82%), it is found only in vertebrates. It can be assumed that the conservatism of cellular receptors and PA is one of the reasons for the lack of resistance to this pathogen in a huge variety of mammal species. It

has been shown that PA83 is a calcium-dependent serine protease, like furin is, and that PA83 is potentially capable to use this activity to bind to TEM8 [43]. PA63 within a complex with the receptor forms oligomers (heptamers or octamers), which bind three or four LF, EF or LF and EF simultaneously. The toxin complex undergoes endocytosis. Under the acidic endosomal pH conditions a number of conformational changes occur in the structure of PA63. This changes lead to the formation of a channel in the endosomal membrane through which LF and EF are transferred to the cytosol, where they manifest their toxicity due to their enzymatic activity [44-46]. Recently, a new function of PA20 has been described and a PA20-mediated mechanism has been proposed, by which insects-carriers of *B. anthracis* gain anthrax resistance due to activation of innate immunity. According to the authors, a similar mechanism may potentially exist in mammals [47].

LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinases MAPKKs, MEKs and MKKs, thus disrupting the activation of signaling pathways of mitogen-activated cascades, including ERK (Extracellular signal-regulated kinase) pathway 1/2, JNK/SAPK (c-Jun N-terminal kinase/Stress activated protein kinase) and p38. Those cascades are crucial for numerous cellular functions such as proliferation and cell cycle regulation, as well as for immunomodulation and survival in toxic strokes [48-50]. EF is a highly effective calmodulin-dependent adenylate cyclase that is about 1000 times more active than adenylate cyclase of mammals and causes a sustained increase in cAMP level. High cAMP concentrations disrupt key cellular functions, leading to negative consequences for the host [51-54].

Nowadays, there is no doubt that anthrax toxins are crucial in anthrax pathogenesis. The release of toxins takes place in early stages after spore germination: PA mRNA is detectable 15 min after the initiation of germination [55]. However, the mechanisms of pathogenesis differ from one animal species to another and depend on the type of disease and other factors. In the initial stages of the infection combined LT and EF activity blocks the host innate immune response. For example, the phenomenon of LT-induced macrophages death is known in some mouse strains. In the later stages, when high LT and ET concentrations are reached, they can cause host death directly through affecting various vital systems, in particular the cardiovascular system and the liver [40, 56]. Experiments on mice have shown that expression of the main toxin receptor CMG2 in vascular smooth muscle cells and in cardiomyocytes is required for the LT-induces lethal outcome. ET-induced mortality is due to disruption of another type of cell, mainly hepatocytes. Targeting endothelial cells with any of the toxins does not contribute to mortality from *B. anthracis* as significantly as it has been considered previously [56]. Thus, both LT and ET are lethal to mice, while each of three toxin components (PA, LF and EF) individually are not toxic [57]. At the same time, experiments on macaques have shown that pathological effects that lead to lethal outcome are mainly due to the activity of anthrax LT [58]. A study of the influence of LT an ET on the subgroups of human alveolar phagocytes and leucocytes with low CMG2 and TEM8 expression has demonstrated that all cell types bound PA in a dose-dependent manner. The cells were invulnerable to LT-induced apoptosis or necrosis at toxin concentrations below 1000 ng/ml. However, exposure to toxins have inhibited spore internalization. Authors suppose that in pulmonary anthrax, ET prevents spore phagocytosis in the initial stages of infection and in the later stages high concentration of LT in the bloodstream suppresses pathogen phagocytosis by leukocytes, thus ensuring rapid *B. anthracis* proliferation in the blood [59]. Perhaps the same mechanism exists in some other animal species. Long-term storage of active enzymes (LF) in endosomal vesicles and their slow release into the

external environment, which is possible even in the absence of bacteria, is an important characteristic of the high virulence of *B. anthracis*. Apparently, this is the reason for lethal outcomes even after a successful elimination of bacteria during antibiotic treatment [60].

Live attenuated vaccines. Anthrax was one of the first bacterial diseases to be controlled by vaccination. The first live attenuated anthrax vaccines were invented by Louis Pasteur in 1881 and have been being used effectively to vaccinate animals in Europe and South America for 50 years. Live attenuated vaccines for veterinary use can be divided into three main categories: Pasteur vaccines, Sterne vaccines and Carbozoo vaccines. The division is based on different mechanism of pathogen attenuation [61]. It used to be assumed that the attenuation according to Pasteur scheme (Pasteur vaccines) leads to the elimination of pXO1 plasmid encoding the main virulence factors (PA, LF and EF), which results in the nontoxigenic encapsulated ($pXO1^-/pXO2^+$) vaccine strain. Currently it is believed that Pasteur vaccines were mixed cultures containing a small percentage of completely virulent bacteria ($pXO1^+/pXO2^+$) [62-64]. Mechanism of attenuation implemented in Carbozoo vaccines is still unknown, however, studies have demonstrated the presence of both plasmids ($pXO1^+/pXO2^+$) in such bacteria strains. These strains are toxigenic and encapsulated [65].

In the 1930s, Pasteur vaccines were driven out by vaccines based on attenuated non-encapsulated ($pXO1+/pXO2^-$) *B. anthracis* Sterne strains. In Sterne vaccines *B. anthracis* lacks pXO2 encoding the formation of capsule components. Worldwide, most anthrax vaccines for animals contain the toxigenic non-encapsulated *B. anthracis* Sterne 34F2 strain obtained from virulent isolate from cattle. In Russia, China and Romania other similar toxigenic non-encapsulated strains are being used [1]. In the USSR, live vaccines based on non-encapsulated STI1 and GNKI strains were used for vaccination in 1940s-1980s, which lead to a significant decrease in the incidence of anthrax in both humans and animals. Since the 1980s the attenuated *B. anthracis* 55-VNIIVViM ($pXO1^+/pXO2^-$) strain has been being used in Russia as a vaccine strain for cattle. In Italy and Argentina, Carbozoo vaccines based on the Italian vaccine strain Carbosap [66] and the Argentine strain A [67] are used to vaccinate animals.

Sterne-like strains (34F2 and its analogues in Russia, China, and Romania) lack the genes for capsule formation while still producing the toxin. These strains are more about to have low virulence than to be avirulent, thus maintaining the capability to cause a certain rate of morbidity [1]. An overdose of vaccines based on these strains is dangerous and can lead to sever consequences up to the death of animals. Some animals, for example goats, are more susceptible to infection and death in such cases, thus requiring a particularly thorough dosage control [1, 14]. It has also been reported that vaccination with the Sterne 34F2 strain can cause the death of miniature horses [68] and llamas [69]. For the Russian strain 55-VNIIVViM, vaccination of horses is recommended only from 9 months of age. It is not recommended to vaccinate pregnant, sick or weak animals. Vaccination is also prohibited less than 6 weeks before slaughtering animals for meat. Other factors limiting the use of currently existing vaccines are the high cost of the drug, the time-limited effect (in epidemically unfavorable regions, the vaccination should be repeated annually), the need for parenteral vaccine administration (a decrease in the protectivity and the appearance of adverse effects are possible even in case of minor deviations from the recommendations on dosage and vaccination regulation). Moreover, the presence of live pathogen spores in the vaccines requires special training of personnel, and all used tanks and glassware should be subsequently sterilized and disinfected [1]. In 2020, the development of a vaccine based on the spores of the microencapsulated, attenuated B. anthracis Sterne 34F2

strain for oral administration was reported. The studies on mice have demonstrated immunogenicity and neutralizing activity against a lethal dose of LT *in vitro* after a single immunization via gavage [70]. This research direction may turn out to be prospective. However, it is desirable to further study the protectivity of microencapsulated spores in farm animals and the safety of such strain for the environment when used to vaccinate wild animals and animals on free-range.

In addition, live attenuated vaccines are not effective enough during anthrax outbreaks, because only 80% of vaccinated animals gain immunity sufficient to resist *B. anthracis* infection 8 days after the first vaccination [71]. Since the immunity develops more than 1 week after vaccination, a long course of antibiotics is recommended before the vaccination. Administration of the vaccine is incompatible with antibiotics: antibiotics should not be prescribed two weeks before and after the vaccination due to their inhibitory effect on the immune response development and to possible anaphylactic reactions [1, 72]. Antibiotics are widely used to treat various disease of farm animals and can be contained in animal feed. This fact also limits the possibilities of effective vaccination against anthrax using live attenuated vaccines [1].

Therefore, the development of an alternative economically efficient vaccine that is safe to use and is compatible with antibiotics is desirable.

Recombinant vaccines. Recombinant vaccines are a promising approach that allows to overcome the limitations of traditional vaccines. Many successfully designed recombinant subunit and vector veterinary vaccines against various pathogens are already used for vaccination [73].

In recent years, recombinant anthrax vaccines for both medical and veterinary purposes have been being actively developed. The potent use of *B. anthracis* spores as a biological weapon has triggered intensive research in the area of creation of new-generation anthrax vaccines for humans. In our review published in «Expert Review of Vaccines» in 2019 it is mentioned that significant efforts were made to develop new approaches to anthrax vaccination and to study recombinant anthrax vaccines being developed [74]. The main direction of modern research in this area is focused on the creation of recombinant anthrax vaccines containing recombinant anthrax protective antigen (PA), which is the main anthrax antigen. PA is a central toxin component and plays a key role in the protection against toxigenic and encapsulated *B. anthracis* strains. Anti-PA antibodies induction is the main immune response after animal vaccination with Sterne vaccine [75-77]. In many studies it has also been shown that vaccines protectivity correlates with PA-induced neutralizing antibodies titer. Most of epitopes, antibodies to which have toxin-neutralizing activities, are mapped to PA [74].

Anthrax vaccines candidates are being developed using various approaches that can be divided into four groups: adenovirus-based vaccines expressing full-size recombinant PA (rPA83) [78, 79]; vaccines based on live bacterial vectors, such as *Lactobacillus* spp., and on attenuated *Salmonella* spp. strains expressing rPA83 [80-84]; DNA vaccines [85-87]; vaccines based on *B. anthracis* recombinant antigens obtained using in vitro expression systems. Most of the vaccines currently being developed are based on the latter approach. Of the 10 vaccines in clinical trials, 8 are created using purified rPA83. Subunit purified rPA83-based vaccines are characterized with high safety and protective properties [74]. In return, subunit vaccine candidates can be divided into three groups: vaccines based in rPA83 obtained in various expression systems [88-92]; vaccines based on chimeric proteins obtained by fusion or conjugation of rPA83m or separate PA domains with additional antigens, such as LF domains [93-97], LF+EF [98), antigenic component of spores [99, 100] or a capsule component — poly-γ-D glutamic acid [101-104], to enhance the immune response; vaccines based on rPA83m mixed with

other components, such as bacterial surface S-layer recombinant protein [105] or *B. anthracis* spores [106, 107]. New approach was implemented during the development of a vaccine based on spores of probiotic *Bacillus subtilis* using a technology that makes it real to effectively express heterologous rPA83 protein in the sporulation phase with further attachment or adsorption of the protein to the spore surface. The effectiveness and safety of such vaccine has been demonstrated for various models of administration, including oral, internasal and sublingual [107]. In addition to these strategies, the possibility of using various adjuvants in order to increase rPA83-based vaccines stability is being investigated [74, 108]. A number of new adjuvants are currently in clinical trials, and data is being accumulated on their effectiveness comparing to aluminum hydroxide, which is an adjuvant that is used in many vaccines [109]. One of the main problems in the development of subunit vaccines based on rPA83 is the instability of this protein. rPA83 contains two sites that are sensitive to proteolytic cleavage. It has also been shown that the instability of rPA83 is associated with spontaneous deamidation of several asparagine residues in the protein and the rate of deamidation increases significantly when aluminum hydroxide is used as an adjuvant. Based on a number of data that has been published recently, it can be argued that rPA83 adsorbed on aluminum hydroxide is unstable and loses its ability to induce neutralizing antibodies during storage [110-112]. New adjuvants together with the use of mutant rPA83 forms that are resistant to deamidation and proteolysis can solve the abovementioned problem. Search for a new adjuvant and rPA83 molecule stabilization are among the main directions of a new effective anthrax vaccine development. The study by Ryabchevskaya et al. [113] has demonstrated the possibility of simultaneous implementation of two approaches to stabilize rPA83 through the adsorption of rPA83 on the surface of spherical particles obtained from a plant virus and through a directed mutagenesis of sites that are a reason for protein destabilization. Thus, over the years, with the development of genetic engineering methods, creation of various vector platforms, DNA vaccines and adjuvant systems used to increase the immunogenicity and the stability of subunit vaccines, there are all required conditions for the creation of new, safer and more effective anthrax vaccines.

Our research on scientific publications devoted to experimental veterinary vaccines with studies on farm animals have revealed only a few articles. However, according to many authors, methods and approaches used for medical vaccines development are also optimal for veterinary vaccines creation. In the study performed by Fasnella et al. [71] rPA83 and mutant forms of LF (mLF-Y728A; E735A) and EF (mEF-K346R) with inactivated enzymatic activity have been cloned and expressed in *Escherichia coli* system successfully. Two vaccine candidates, monovalent containing PA83 and trivalent (TV) containing PA83, mLF and mEF were used to immunize rabbits in the presence of Marcol 52 (ESSO) and Montane 80® (SEPPIC) adjuvants, commonly used as adjuvants for veterinary use. New Zealand rabbits single time immunized subcutaneously with rPA83 and TV have produced high levels of antibodies against PA (rPA83 and TV vaccines), LF and EF (TV vaccine), and both vaccines have demonstrated 100% protection of rabbits against virulent *B. anthracis* strain 0843 (200LD50) 1 week after vaccination. Sterne-vaccinated rabbits have demonstrated lower anti-PA, anti-LF and anti-EF antibodies level comparing to those induced by experimental vaccines. Also, when tested under the same experimental conditions, Sterne vaccine protected only 80% of infected rabbits 1 week after vaccination. Thus, both vaccine candidates have been proven to be more effective than Sterne vaccine. The possibility to use them with antibiotics simultaneously due to the lack of a live pathogen in their composition is another significant advantage of these vaccine candidates. However, it should be mentioned that advantages of the trivalent vaccine over the vaccine containing

a single rPA83 antigen have not been shown. Moreover, there is data showing that the use of catalytically inactivated through a single spot mutation mutant EF in conjunction with PA may be unsafe [114].

In the study by Koehler et al. [115] the immunogenicity of two recombinant multicomponent vaccines has been evaluated in goats using a lipopeptide adjuvant. These vaccines have contained rPA and an antigenic spore somponent BclA (bacillus collagen-like protein of anthracis) expressed in *E. coli* systems alongside with spores inactivated with formalin (FIS). Goats are extremely susceptible to *B. antracis*. Goats, three times subcutaneously immunized with rPA+rBclA or rPA+rBclA+FIS have demonstrated 50% and 80% protection, respectively, against a lethal dose of virulent *B. anthracis* strain spores. Further studies performed by the same group of scientists have demonstrated that immune serum from goats vaccinated with rPA+rBclA or rPA+rBclA+FIS is capable to protect approximately 70% of mice against lethal dose of anthrax spores [116]. Thus, the experiments carried out have demonstrated the efficacy of two recombinant anthrax vaccines and the induction of a protective immune response in vaccinated goats. Preliminary data from serological studies on goats have confirmed the reliability of immunogenicity of these vaccines when administered simultaneously with antibiotics.

Currently, the same scientific group continues their studies on the vaccine candidate in cattle. Jauro et al. [106] have compared the immunogenicity and protectivity of a vaccine candidate based on rPA83 combined with FIS and with aluminum hydroxide-containing adjuvant (Emulsigen-D/Alhydrogel) and a vaccine based on the spores of an attenuated Sterne 34F2 strain. After the vaccination of cows the immunogenicity and protectivity *in vitro* in a toxin-neutralizing test and *in vivo* in a mouse model with passive immunization have been evaluated. It has been demonstrated that the antibody titers were similar in case of both vaccines. However, it has been shown that, in contradistinction to Sterne vaccines, vaccine candidate is effective when used simultaneously with antibiotics [117].

Several scientific groups implement various approaches to rPA83 obtaining in a plant expression system [74]. Gorantala et al [118] have attempted to develop a universal oral vaccine suitable both for veterinary and medical use. To achieve this aim, transgenic mustard plants (*Brassica juncea*) have been obtained for the expression of rPA83, the leaves and stems of these plants can be used fresh for human consumption and for cattle feeding. In addition, flour obtained from *B. juncea* is used as feed for cattle in many countries [118]. The presence of standard transformation protocols for *B. juncea*, large plant biomass, long-term stability of the transgene, and safe storage of the antigen in seeds indicate that transgenic mustard plants can effectively express rPA83 and become the basis for the development of a vaccine against anthrax. In has been shown that transgenic *B. juncea* plants after repeated oral administration to mice for 1 month with a final booster dose of rPA83 obtained in *E. coli* (also administered orally) in the presence of mucosal adjuvant have stimulated both systemic and mucosal immune responses. Also, 60% of the mice have survived the lethal dose of *B. anthracis* (Sterne strain). In control experiments using rPA83 from *E. coli* being administered orally (by gavage) 80% of the mice have survived the lethal dose of *B. anthracis*. The need for rPA83 booster dose may be due to the low protein expression level in transgenic mustard (0.3-0.8% of a total soluble protein fraction) [118]. According to the authors, further research on improving the protein expression is required. In particular, the creation of transplastomic mustard plants with PA83 gene integrated into the chloroplast genome is proposed. In the same study, transplastomic tobacco plants have been obtained with rPA83 expression levels equal to 2.5-4% of the total soluble plant protein fraction. In addition, the authors believe that the

peculiarities of the digestive processes in ruminants can contribute to the effective impact of the antigen on the lymphoid tissues associated with the intestine, and thus enhance the immune response of the intestinal mucosa. As already mentioned, after entering the organism spores germinate turning into a vegetative form locally at the primary pathogen-host contact sites, for example, in the intestinal mucosa. Therefore, the development of an oral recombinant veterinary vaccine in a prospective and important research direction.

In another study, transplastomic tobacco plants have been obtained in which the level of rPA83 expression in mature leaves has reached 14.2% of the total soluble plant protein fraction mass. Calculations have shown that one acre (0,405 ha) of transplantomic tobacco plants expressing rPA83 is capable to produce up to 360 million doses of anthrax vaccine [119]. The expression of anthrax antigens using plant expression system is a promising direction for vaccination of free-range ruminants. This direction, undoubtedly, deserves further development.

So, the particularities of the life cycle and ecology of the anthrax causative agent indicate that it is not yet possible to eliminate this disease completely. Vaccination is the main way to combat anthrax. Live attenuated vaccines that have been being used for decades are effective but are also characterized with several limitations. In particular, they are incompatible with antibiotics, which are necessary both during anthrax outcomes and for the treatment of other animal diseases. Therefore, the use of recombinant vaccines that can be administered simultaneously with antibiotics is extremely relevant and, moreover, in fact is the only promising way to improve epizootic and epidemic situation with this dangerous disease. The central direction of modern anthrax vaccine development is focused on the creation of subunit vaccines containing the sequence of protective antigen (PA) — the main anthrax toxin antigen. The undoubted priority is given to the creation of safe and effective vaccines for humans, but the same approaches and antigens are potentially suitable for vaccination of farm animals. Recombinant vaccines, including those for oral administration, is a prospective direction of the development of veterinary vaccines. On farm animals, data has been obtained on the effectiveness of vaccine candidates based on PA83 mixed with inactivated *B. anthracis* spores, and it has been proven that, in contradistinction to Sterne vaccine, the vaccine candidate is highly effective when administered simultaneously with antibiotics. Subunit PA83-based vaccines are promising, both vector ones with *in vivo* expression and ones based on recombinant antigen obtained *in vitro* and stabilized without loss of immunogenicity. In our opinion, mutant forms of rPA83 that are resistant to proteolysis are promising when combined with new adjuvants and/or carrying platforms. Another important direction of recombinant veterinary vaccines development, which requires attention, is the use of probiotics (*Lactobacillus* spp.) as vectors for the delivering of the antigen or for the antigen exposing on the surface of *B. subtilis* spores with the possibility of oral administration.

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INTERACTION OF NUCLEIC ACIDS WITH MOLECULES OF WATER, PROTEINS, AND INTERCALATORS

(review)

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Abstract

Modern concepts of intermolecular interactions in the cell are incomplete without understanding how complexes are formed between nucleic acids and the main intracellular components — water and proteins, and what determines the spatial stabilization of such complexes. The same is true for intercalation — intracellular intermolecular interaction of planar structure substances capable of being introduced between adjacent pairs of nitrogenous bases into DNA and RNA molecules, which plays a special role in pharmacology and genetic mutagenesis. In addition, intercalation can have a strong effect on cellular metabolism, slowing down and in some cases stopping the growth of cells, which, under certain conditions, leads to both apoptosis and cancer, or vice versa, to the body's recovery from such diseases (M. Ashrafizadeh et al., 2020). This review is devoted to the consideration of molecular mechanisms and the biological role of these processes. It is known that the DNA double helix can interact with polypeptides through the formation of specific hydrogen bonds between Watson-Crick base pairs and amino acid side chains (C.N. Pace et al., 2004), through intercalation of aromatic amino acid side chains between base pairs, at which some specificity is also manifested (A. Bazzoli et al., 2017), and due to the direct binding of protein α-helices and β-layers in DNA grooves (E. Del Giudice et al., 2009). It is assumed that the latter type of interaction takes place, for example, in DNA complexes with the *cro*-repressor of gene expression and with a protein that activates catabolism, for which two models of the binding of α-helices with the left-sided and right-sided DNA double helix in the B-form have been proposed. It is indicated that if the structure of a nucleic acid molecule is known, then the size of the surface of DNA and RNA available for water molecules or other solvents can be calculated. In the case of DNA folding in solution into a double helix, its molecule becomes polar. With this kind of hydration, two hydration shells are formed around the DNA molecule. The first of them, consisting of ~ 20 water molecules per nucleotide, is impermeable to cations and does not resemble ice in its aggregate structure, while the second shell is indistinguishable from ordinary water. Differences in the structure of hydration shells shed light on the nature of the conformational transition between the B → A forms, which occurs with a decrease in the hydration of the DNA molecule. The interaction of nucleic acids with molecules of medicinal and other planar substances is also described. At the same time, the review considers only intercalation complexes with drugs whose molecules have a planar structure or have planar functional groups. It has been demonstrated that the binding of such substances with a double helix proceeds in two stages: at the first stage, they are attached along the periphery of the helix, at the second, intercalation occurs, that is, the actual insertion of the intercalator in the planar plane between nucleotide pairs. This kind of intercalation is accompanied by unwinding and elongation of the nucleic acid helix, as well as an increase in its rigidity. In accordance with the principle of exclusion of the nearest binding sites, according to which it does not occur at each nearest neighbor along the axis of the DNA double helix due to spatial constraints, which are determined by the stereometry of nucleotides adjacent to intercalators, intercalator molecules fill only half of such places. In general, the interactions of nucleic acids with water molecules, proteins and intercalators described in the work indicate the biological significance of this kind of relationship, since, as is known, the stability and regularity of the processes of replication and expression of genes plays an important role in the genotype—environment interaction and the «implementation» of genetic information at the molecular level.

Keywords: nucleic acids, A-DNA, B-DNA, conformational transitions, water molecules, DNA hydration, proteins, ligands, planar intercalators, intermolecular interactions, replication, gene expression

Modern intermolecular interactions at the intracellular level are not complete without considering the creation and stabilization of spatial complexes formed by nucleic acids and such basic intracellular elements as water and proteins. Water is the main element in which all bioorganic components and substances are dissolved inside the cells of a living organism. Water is a highly polar solvent and essential for all living organisms [1-3]. Nucleic acids are very soluble in water [4, 5]. All the main biochemical processes take place in it, due to which the role of water in the chemical structure of living organisms cannot be overestimated. As a reagent, water takes part in many chemical reactions. For example, the hydrolysis of proteins, fats and carbohydrates occurs with the direct participation of water, and during the hydrolysis of ATP energy is released, which is necessary for the implementation of energetically unfavorable enzymatic reactions [6-9]. In a liquid state water is practically not compressed, and as a result, serves as a kind of skeleton for the cell. Water molecules are characterized by cohesion, which, in turn, characterizes the strength of the body ability to withstand external influences. Due to its osmotic properties, water creates excess pressure inside the vacuoles of plant cells [10]. This turgor pressure allows the cell wall to maintain its elasticity and maintain the shape of organs (e.g. leaves). At the same time, water, being an electrically neutral molecule, has a small positive charge in the region of hydrogen atoms, although it is unevenly distributed inside the molecule. However, in the region where the oxygen atom is located, the charge is slightly negative. Due to this structure, water molecules can interact with each other and with other bioorganic molecules inside cells through the so-called hydrogen bonds [11, 12]. All this determines the importance of water as one of the main elements of the cell during its functioning, including the implementation of genetic information at the molecular and cellular level.

Another element that plays a critical role in the cell is proteins [13, 14]. Proteins play not only a building role inside cells, but also allow metadata to be realized, encoded in the main carrier of genetic information - nucleic acids. Proteins are nitrogen-containing high-molecular organic compounds, the monomers of which are amino acids. Proteins are characterized by a complex spatial structure that has primary, secondary, tertiary and quaternary levels of organization. In order to carry out their biological functions, proteins take one or more specific spatial configurations due to such non-covalent interactions as hydrogen, ionic, hydrophobic bonds and other intermolecular interactions [15-18]. The complexity of the structure of protein molecules is associated primarily with the variety of their functions. At the same time, the structure of molecules depends on the properties of the environment and intermolecular interactions. Protein interactions with nucleic acids play a special role in the life cycle of any living organism, since it is they that ensure the constancy and reproducibility of the genetic information encoded in DNA and RNA. However, as practice shows, the spatial stereochemical mechanisms of interaction between proteins and nucleic acids have not been fully elucidated, which is especially important in the light of understanding and the possibility of controlling this kind of intermolecular interactions [19-21].

Another intracellular intermolecular process that attracts some attention, and which plays a special role in pharmacology and genetic mutagenesis, is intercalation [22, 23]. There are several ways in which certain molecules, commonly called ligands, can bind to DNA. Ligands usually either intercalate into the DNA molecule by inserting in the plane between the bases of the double helix, or bind to it electrostatically or covalently. In order for intercalation to occur, the ligand must have the appropriate size and chemical nature. Typically, intercalating ligands are flat and have an aromatic polycyclic structure. In chemotherapy pharmacology, intercalating ligands are used as agents that inhibit DNA replication to

stop the growth of cancer cells. For example, daunorubicin and adriamycin are used to treat Hodgkin's disease [24, 25], for the treatment of Ewing's sarcoma - dactinomycin [26]. The antibiotic actinomycin D, intercalating between adjacent pairs of DNA nitrogenous bases, restricts the binding of RNA polymerase to the DNA matrix and thereby prevents the interaction of the enzyme with the DNA strand [27]. In molecular biological studies, intercalating ligands are used for fluorescent staining of nucleic acid molecules or for mutagenesis. For example, ethidium bromide is typically used in nucleic acid electrophoresis in agarose or polyacrylamide gels [28], and acridine orange or acridine yellow generate cause chromosomal mutations such as deletion, leading to the loss of the middle part of chromosomes, or for in vivo staining of cell nuclei or for the analysis of biological membranes [29, 30].

In this review, we briefly consider the intermolecular mechanisms of interaction of nucleic acids with water, proteins, and intercalating substances. The importance of such interactions, which play a key role in the stability and regularity of the processes of replication and expression of genes in the practical implementation of the "genotype—environment" interaction, as well as the implementation of genetic information at the molecular level, as well as in ensuring the constancy and reproducibility of the genetic information.

Water and nucleic acids. Needless to say, how important is the role of the aqueous environment of nucleic acids. Water is not just a medium in which certain molecules are dissolved. Water interacts with dissolved molecules, and it is this water that mainly stabilizes the secondary and tertiary structure of macromolecules [11, 31, 32]. This applies to both proteins and DNA, and DNA, perhaps even more so, since the high dielectric constant of water and hydrated counterions weaken the electrostatic repulsion of phosphates [33-35]. Water molecules also take part in the process of self-assembly of bases into ordered structures, since this process is largely due to hydrophobic interactions. The degree of DNA hydration is of decisive importance for its conformation: at high relative humidity, DNA is in the B-form (fig. 1), a decrease in moisture (or an increase in ionic strength) leads to the transition of DNA from B- to C-, A- or (if the sequence allows) into D- and Z-forms [32, 36].

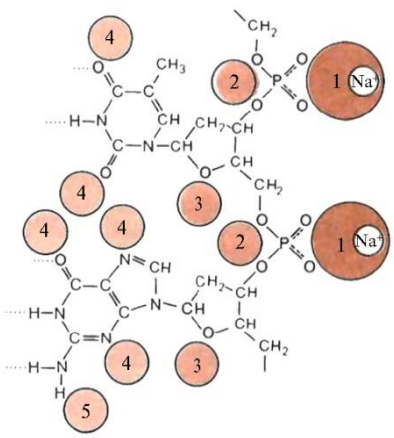

Fig. 1. Sites of preferred binding of water molecules to B-DNA (on [42]). The numbers from 1 to 5 indicate the order in which the bond strength decreases. There are about 5 water molecules near the phosphate group.

Hydration of nucleic acids plays an important role in the formation of their structure and is responsible for A ↔ B transitions in DNA [37, 38]. Two hydration shells are formed around the DNA molecule. The first of them, consisting of ~ 20 water molecules per nucleotide, is impermeable to cations and does not resemble crystal water in structure; on the ice. The second shell is indistinguishable from ordinary liquid water. If the structure is known, then the surface of DNA or RNA accessible to solvent molecules can be determined by calculation. The results of such calculations explain some of the features of the behavior of DNA in solution and, in particular, show that when folding into a double helix, the DNA molecule becomes more polar. X-ray diffraction analysis of single crystals of oligonucleotides showed that in the regions consisting of AT-pairs, in the minor

groove of B-DNA, a ridge is formed of water molecules hydrogen-bonded to bases [39]. In A-DNA, water "strands" are formed that cross-link the phosphate groups lying at the edges of the main groove. Such differences in the structure of hydration shells shed light on the nature of the B ↔ A transition that occurs with a decrease in the water content in DNA. In crystals of some cyclodextrins and d(CpG) complex with proflavine, four-, five-, and six-membered cyclic structures are found that form hydrogen-bonded oxygen atoms or O-H groups [40, 41]. It is possible that such structures are included in the hydration shells of macromolecules.

At high water activity, when cations do not violate the primary hydration shell, consisting of 20 water molecules per nucleotide, DNA is in the B-form. As the relative humidity in the fiber or film decreases or as the salt concentration in the solution increases, the degree of hydration also decreases at a certain threshold value G (a parameter that determines the solvation or hydration of a macromolecule, which in the case of DNA is equal to the number of moles of water per mole of nucleotides), corresponding to about 20 water molecules per nucleotide, a structural transition of DNA from the B-form to the C- or A-form is observed, depending on the nature of the counterion present. B → C-transition occurs “continuously” [43, 44], as it should be in the case of two structurally similar forms. As for the transitions B → A and C → A, because of the change in the conformation of the sugar $C_{2\text{-}endo} \rightarrow C_{3\text{-}endo}$, they should occur in a jump, cooperatively.

In aqueous solutions, B-DNA is slightly untwisted, its spiral rotation angle is less than that of B-DNA in fibers. However, as the salt concentration increases, there is a “continuous” intrafamily structural transition B ↔ C. When the salt concentration reaches a certain value, there is a sharp cooperative interfamily transition C → A or B → A. Such cooperative transitions (B → A or C → A) also occur when the polarity of the medium changes, for example, when ethanol, isopropanol or dioxane are added to the system to concentration ~ 80% [45-47]. When the double helix is formed, the DNA becomes more polar. In other words, if in the unwound elongated DNA, phosphates account for ~ 820% of the surface area, bases for 50% and sugars for 30%, then in double helix phosphates occupy 45% of the surface area, bases 20%, and sugars 35%. Thus, the polarity of the DNA molecule increases during the formation of a double helix. The same picture is observed in proteins during the formation of globular structures: polar groups are located on the surface, and non-polar, hydrophobic ones are inside. In general, we can say that the accessibility of the DNA surface for a solvent determines its properties. Estimates of the accessible surface area of DNA show that the bases of B-DNA are open mainly from the side of the main groove, and the bases of A-DNA are open from the side of the minor. This means that when a protein interacts with an intact double helix, specific contacts between base pairs and side groups of amino acids in the case of the B-form will occur in the main groove, and in the case of the A-form, in the minor. This is consistent with data on the interaction of B-DNA with specific proteins, the *Escherichia coli* RNA polymerase with a *lac*-promoter, a *lac*-repressor with a *lac*-operator, as well as with data on λ- and *cro*-repressors and the interaction of DNA with histones in nucleosomes [48-50].

Interaction between proteins and nucleic acids. The interactions between proteins and nucleic acids occur at all stages of DNA replication and expression, as well as in the course of numerous regulatory processes, and, therefore, their role is extremely important. Nevertheless, our knowledge of the molecular mechanisms of such interactions is still limited. We do not have a clear idea of how restriction endonucleases bind to DNA and cut it at specific locations. There is no complete information about the geometry of recognition of the operator sites by the repressor and how aminoacyl-tRNA synthetases recognize “their”

tRNAs [51]. The main difficulty in studying these complex systems is that it is necessary to simultaneously observe each of the interacting macromolecules. At the same time, spectroscopic methods, with rare exceptions, give inadequate results, and crystallization of complexes of proteins with nucleic acids is associated with many difficulties. Nevertheless, in the early 1980s, publications appeared the authors of which managed to obtain specific complexes of proteins with DNA [52, 53] and tRNA [54] in a form suitable for X-ray structural analysis.

To simplify the process of studying such complex systems, studies of model compounds are carried out using both theoretical and experimental approaches. The main goal of such studies is to establish the specificity of recognition of four types of nucleic acid bases by side groups of twenty amino acids. In this case both monomeric components of both partners and polymers, in some cases synthetic, are used. The binding of nucleotides (inhibitors or coenzymes) in the active centers of enzymes is also studied. Crystals of several proteins that recognize specific DNA sequences were obtained and their spatial structure was determined. This is perhaps the maximum that can be done without crystals of specific DNA-protein complexes. Such structures give an idea of the general principles of nucleic-protein interactions, but the last word ultimately remains with the studies of the complexes themselves [55, 56].

If we carefully analyze the structure of the side groups of amino acids and the polypeptide backbone, we will see that there are four potentially possible types of interactions between proteins and nucleic acids [57-59]: 1) salt bridges between phosphates and positively charged amino acid groups (N_ξ-amino group of lysine, guanidine group of arginine and protonated His residue); 2) hydrogen bonds between phosphates, sugars, nucleic acid bases and peptide groups or hydrophilic side chains of amino acid residues; 3) stacking interactions between side groups of aromatic amino acids (Trp, Tyr, Phe, His) and bases; 4) hydrophobic interactions between nucleic acid bases and side groups of non-polar amino acids.

The energy of these four types of interaction generally decreases in the order in which they are listed here. Since the attraction of opposite charges plays the main role, data on the charge distribution in the side chains of amino acids and in the peptide group are extremely important. They need to be compared with similar data for nucleic acid components and then some interactions can be predicted. However, in reality, a more complex situation is observed, since numerous weak interactions can suppress specific interactions of the "charge-charge" type.

Double helix DNA can interact with polypeptides in several ways: (a) through the formation of specific hydrogen bonds between Watson-Crick base pairs and amino acid side chains; (b) by intercalation of side chains of aromatic amino acids between base pairs ("bookmark" model), which also exhibits some specificity, (c) by direct binding of protein α-helices and β-sheets in DNA grooves. It is assumed that the latter type of interaction (it is more correct to say "two types of interaction") takes place in DNA complexes with a *cro*-repressor (in the model of the complex, the α-helix and β-sheet fall into the major and minor grooves of the double helix, respectively) and with a protein that activates catabolism, for which two models of binding of α-helices have been proposed, i.e., with the left and right double helix of B-DNA [60]. Based on the data on the crystal structure of prealbumin, a hypothetical complex of prealbumin with DNA was proposed [61]. However, in reality, such a complex, apparently, does not form [62]. Both polylysine and polyarginine bind irreversibly to DNA; in both cases, the process of joining is cooperative, but the structure of the corresponding complexes is different.

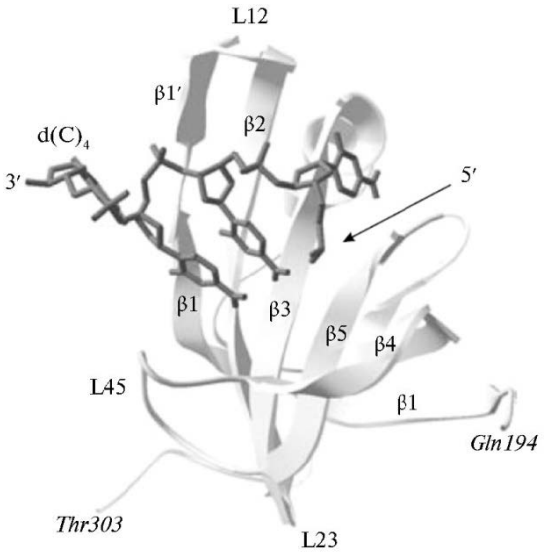

Fig. 2. An example of "straight polarity" binding of nucleic acids in the tertiary structure of the complex of the OB-domain p70A (a.b. 194-303) **hsRPA with the oligonucleotide residue d(C)4** (highlighted in dark gray) (on [63]).

Another example of "straight polarity" binding of nucleic acids in the tertiary structure of the OB-domain complex (the domain that binds oligosaccharides and oligonucleotides) p70A hsRPA with the oligonucleotide residue d(C)4 (Fig. 2). The resulting β-sheets are arranged orthogonal to each other and form a β-cylinder with the chain topology β1-β2-β3-β5-β4-β1. Usually, the canonical surface of interaction of the OB-packing with ligands is located in the region of chains β2 and β3. Additionally, the interaction can involve loops between β1 and β2 (loop L12), β3 and α (loop L3a), α and β4 (loop La4) and, finally, between β4 and β5 (loop L45). These loops form a trough that runs along the domain surface perpendicular to the axis of the topological β-cylinder [63].

Nucleotides and single-stranded RNA and DNA, when bound to proteins, usually take on an elongated shape, i.e., the torsion angle γ for them lies in the region *-ck* or *an*. Such a change in the γ angle occurs when NAD+ binds to dehydrogenases, when dinucleotide phosphates bind to ribonucleases A and S, and when RNA binds to the tobacco mosaic virus protein [64]. Interactions in the complex can represent contacts of any type and affect any part of the molecule of each partner. For example, in the case of a specific complex of ribonuclease T1 with guanylic acid, guanine is recognized through the formation of a hydrogen bond with the peptide backbone and stacking with the tyrosine side group. It turned out that interactions between nucleotides and atoms of the protein backbone are generally more common and more specific than interactions with side groups of amino acids [65].

Binding of DNA to the phage fd gene 5 protein promotes the divergence of the strands of the double helix. If (single-stranded) DNA and protein form a complex, i.e., if DNA is attached to the "active center" of the protein, which consists exclusively of β-structure elements, the formation of protein aggregates begins. In this case, the protein molecules line up one after the other, forming a spiral onto which single-stranded DNA sections are wound [66].

Intercalation. DNA, as a carrier of genetic information, interacts with many drugs, carcinogens and mutagenic substances, as well as with dyes, a characteristic feature of which is the presence of elongated (hetero)cyclic aromatic chromophores. Such substances include: acridines (yellow and orange), proflavine, ethidium, ellipticine, 3,5,6,8-tetramethyl-N-methylphenanthroline, 2-hydroxyethanethiolate-2,2',2''-terpyridine-platinum (III), daunomycin, actinomycin and a number of others (Fig. 3). Since DNA plays a key role in the processes of replication and protein biosynthesis, its modification when interacting with these compounds has a strong effect on cellular metabolism, slowing down and in some cases stopping cell growth. All these properties of the compounds mentioned above

aroused great interest in them, especially increased over the past decades. The possibility of their use in medicine was discovered; they also found wide use in laboratories in the study of the structure and function of DNA [67, 68]. One of the categories of such compounds is formed by substances that lead to chemical modification of DNA (and the target is primarily guanine), the other is those that bind to the double helix. Binding occurs either at the periphery of the molecule, or, as, for example, in the case of drugs such as daunomycin and actinomycin, by intercalation (i.e., by incorporation) between adjacent base pairs without breaking Watson-Crick pairing (Fig. 4). The interaction of DNA with medicines is of great importance for pharmacology [69]. If we consider only intercalation complexes with drugs with planar groups, then the binding of such drugs with a double helix occurs in two stages: at the first, they are attached along the periphery of the helix, at the second, intercalation occurs, which is accompanied by unwinding and lengthening of the helix, as well as an increase in its rigidity. In accordance with the principle of exclusion of the nearest binding sites, intercalator molecules fill only half of such sites.

In all known crystalline complexes, intercalators are located between two Watson-Crick pairs formed (in the case of ribo- and deoxyriboside) by self-complementary dinucleoside monophosphates, in which the nucleoside at the 5′-end is always pyrimidine, and at the 3′-end-purine. If the bases are reversed, then either crystals are not formed, or the complex will have a non-helical structure. This specific sequence dependence of intercalation was also found for aqueous solutions. It also received a theoretical basis. It was shown that for both DNA and RNA upon intercalation into the pyrimidine-3',5'-purine sequence, much stronger base overlap with the intercalator occurs than upon intercalation into the purine-3',5'-pyrimidine sequence [70]. Therefore, in the first case, intercalation turns out to be 7-13 kcal mol^{-1} more favorable than in the second [71]. This intermolecular interaction is stabilized by intramolecular electrostatic forces, which also provide preferential insertion into the pyrimidine-3′,5′-purine sequence. In addition, in the case of an inverted sequence, sterically unfavorable contacts may occur, which complicate the integration.

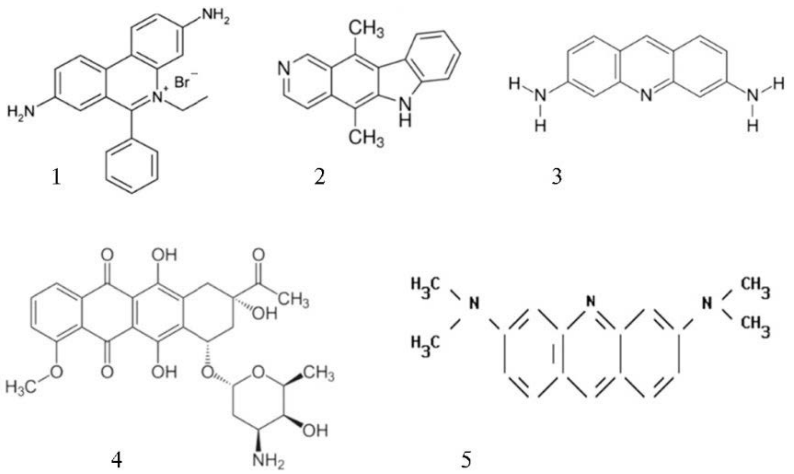

Fig. 3. Examples of some medicines and dyes that form complexes with nucleic acids by the type of intercalation: 1 — ethidium bromide, 2 — ellipticine, 3 − proflavine, 4 — daunomycin, 5 — acridine orange.

During intercalation, the physical properties of double helices change. For the first time, the assumption about the possible incorporation of planar aromatic

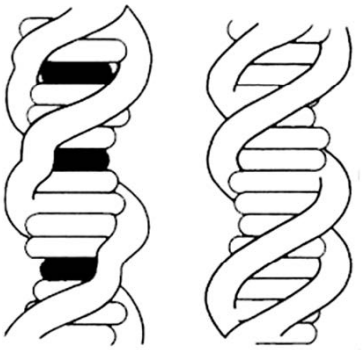

Fig. 4. Intercalation of planar molecules of intercalating substances (black plates) **into the DNA double helix.** The regular course of the sugar-phosphate backbone (right) in the intercalation sites is disrupted (left).

molecules between neighboring base pairs was made on the basis of the results of hydrodynamic and X-ray structural studies of DNA in the presence of acridine dyes [72]. If you add an acridine dye to the DNA solution, and then prepare the fiber and irradiate it with X-rays, you will get an X-ray diffraction pattern in which the reflections due to the presence of a regular helical structure blur and disappear. Only equatorial reflections remain, indicating a regular packing of molecules along the fiber, and a strong meridional reflex (0.34 nm), which corresponds to the interplanar distance between pairs [72, 73]. Generally speaking, when the aromatic molecules are embedded with a thickness of 0.34 nm, the overall stacking scheme may not be violated. However, for intercalation to occur, the pairs must move apart, and this will lead to a change in the geometry of the sugar-phosphate backbone and the destruction of the regular helical structure (see Fig. 4). In accordance with this concept, a DNA molecule should elongate as a dye is added to the system. This is precisely what the experimental data prove that the viscosity increases and the sedimentation coefficient decreases [72]. Both of these effects also suggest an increase in the rigidity of the DNA double helix.

Intercalation leads to unwinding of the DNA molecule. The base pairs must move apart to make room for the intercalator. This is carried out due to the simultaneous stretching of the B-DNA double helix along the axis of its untwisting, which is necessary so that the sugar-phosphate backbone does not break during stretching [73]. The fact that unwinding actually occurs is evidenced by the results of experiments on the intercalation of a number of compounds into plasmid DNA, a circular closed double helix that forms a right supercoil [67, 74]. During intercalation, unwinding of the double helix is observed (10-20° for each intercalated molecule), at which the number of right-handed supercoils decreases until equilibrium is reached, i.e., until DNA becomes a simple ring without supercoils. Upon further intercalation, the double helix continues to unwind, and as a result, a left super helix is formed. The process is easy to observe by the change in the sedimentation coefficient [75].

In our experiments, the effect of intercalator dyes and laser radiation was observed both for unwinding double helices of native phage and high molecular weight plant DNA, and for directed formation of single- and double-stranded breaks to increase the frequency and spectrum of recombination in order to release selectable genotypic variability in various plant species [76-79]. As a result, it was found that dyes-intercalators form a stable complex, which, when irradiated with laser light, transfers energy from the donor-intercalator to the DNA acceptor, which leads not only to unwinding of double DNA strands, but also to the formation of single- and double-stranded DNA breaks. At the same time, the DNA structure in places other than intercalation does not change and retains the ability to replicate, and intercalating dyes are able to inhibit nuclease activity. Thus, not only a mutagenic, but also a recombinogenic effect is achieved, which leads to the induction of genotypic variability in plants and can be used in practical genetic selection studies.

It is noteworthy that during intercalation into A-type double helices, all sugar residues can remain in the $C_{3\text{-}endo}$-conformation. The divergence of pairs is mainly due to a change in the conformational angles of the framework ζ and γ,

the values of which are shifted to the region an; in this case, the process proceeds without untwisting and the complexes of proflavine with CpG and with 5-iodine-CpG are apparently acceptable [80, 81]. Since the conformation and orientation of sugars do not essentially change, it can be assumed that the principle of exclusion of the nearest binding sites is not fulfilled during intercalation in A-DNA and A-RNA.

In the case of B-DNA, the main natural form in which, upon intercalation, the angles α and γ shift to the *an* region, a different picture is observed. In the best model from the point of view of intercalation [82] the conformation of the sugar located on the 5'-side of the intercalation site changes from $C_{2'\text{-}endo}$ to $C_{3'\text{-}endo}$ in accordance with what was observed for complexes of dinucleotide monophosphates with intercalators. Conformational changes are not limited to the violation of the position of two pairs adjacent to the intercalator, but extend to other, distant pairs. The 18° unwinding associated with the incorporation of each molecule of the intercalator affects at least three pairs before and after the intercalation site.

This "long-range action" can be clearly seen, for example, in the complex of daunomycin with hexadeoxynucleotide [83] and is consistent with the principle of excluding closest binding sites. Experiments on dichroism have shown that the plane of the intercalator is not perpendicular to the axis of the spiral, but is inclined to it at an angle of about 20°. This affects the location of both the closest pair and the pair following it, i.e., in this case, there is a cooperative effect [84].

Therefore, the presented data allow for the conclusion that the interactions between proteins and nucleic acids are very diverse: these can be salt bridges, hydrogen bonds, stacking and hydrophobic interactions. Any part of both molecules can be involved in them. Molecules of nucleotides, single-stranded DNA and RNA usually elongate when bound to proteins.

Water is not just a medium in which certain molecules are dissolved. Water stabilizes the secondary and tertiary structure of macromolecules. This applies to both proteins and DNA, and to DNA, perhaps even more so [85, 86]. Nucleic acid hydration, as mentioned above, plays an important role in the formation of their structure and is responsible for the A ↔ B conformational transitions. It should be noted that the B-form is the main natural form in which DNA is in the cell and interacts with intracellular proteins, ensuring the activity and replication of encoded genetic information.

When folding into a double helix, the DNA molecule becomes more polar, and this can determine intercalation of flat substances into a double strand of nucleic acids. Intercalation of planar medicinal and other chemical substances or their planar groups is of great importance not only for pharmacology [77, 78]. It also affects replication, including inhibition of nuclease activity, and can also be used for directed mutagenesis and recombinogenesis [76, 79]. Intercalation has a key effect in the modification of DNA during replication and protein biosynthesis [87, 88]. Structural modifications due to DNA interaction with intercalators strongly affect cell metabolism leading to slowing down and even stopping cell growth which can result in apoptosis and malignancy or, conversely, in recovery from cancer diseases [89, 90]. Understanding the mechanisms of such interactions elucidates the role of each intercalator in order to solve practical problems of genetics, physiology, and pharmacology. For example, preparations with a planar structure, containing a Pt chelate complex with bipyridine, strongly change the DNA structure, which takes the form of a "rope ladder" [91]. Moreover, in each Watson-Crick pair, one nucleotide is in *syn*-form and the other in *anti*-form, like in Z-DNA.

So, intracellular interactions of nucleic acids with water and protein molecules are extremely important, since this fixes and stabilizes the spatial structure of DNA and RNA at the molecular level, which, in turn, ensures the stability and regularity of replication processes and gene expression, especially under genotype-environment interactions. Water stabilizes the secondary and tertiary structure of the macromolecules dissolved in it. This is extremely important for DNA, since the conformational changes of its molecule and, ultimately, the functional activity of DNA as a carrier of genetic information depend on the degree of hydration. The interaction of DNA with intercalator drugs is also of great importance. Intercalation, influencing replication and protein biosynthesis, affects cell metabolism, which under certain conditions can have a therapeutic effect. It is reasonable to hope that the study of the interactions of nucleic acids with water and with proteins at the intracellular level will soon disclose mechanisms of nucleic acid-protein recognition and thereby allow us to come close to managing such interactions. The study of the complexes themselves that are formed by nucleic acids with water, proteins, and intercalating agents are also highly informative.

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LARGE-GRAINED WHEATGRASS VARIETY SOVA (*Thinopyrum intermedium*) AS AN ALTERNATIVE TO PERENNIAL WHEAT

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Abstract

For the last decades, due to climate warming, environmental threats, increasing of energy intensity of the grain production, wider usage of perennial cultures as an alternative to annual agricultural cultures, more resistant to negative biotic and abiotic environmental factors has been proposed. The large-grained wheatgrass variety Sova was created at Omsk State Agrarian University via mass selection of wintered biotypes from the population of *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey obtained from The Land Institute (Kansas, USA), with following targeted cross-pollination and creation of new winter-hardy population. In 2020, the variety was registered in the State register of breeding achievements approved for use for all regions of the Russian Federation. For the first time, the biological and economical significance of the large-grained wheatgrass variety Sova as an alternative to perennial wheat is presented in this work. The variety Sova of *Thinopyrum intermedium* is recommended for cultivation as grain and fodder crop during four-six years, the variety forms grain with high protein content and good quality hay. The goal of this research is evaluation of economically valuable traits of new large-grain wheatgrass variety Sova under conditions of southern forest-steppe of Western Siberia, as well as to determine the correlation of spike components with plant height for increasing of the selection efficiency and thousand kernel weight. The research was carried out in the experimental field of Omsk State Agrarian University under conditions of the southern forest-steppe of Western Siberia in 2015-2019. A new wheatgrass variety Sova (*Thinopyrum intermedium*), winter bread wheat (*Triticum aestivum* L.) cv. Omskaya 4, and spring bread wheat (*T. aestivum* L.) cvs. Element 22 and Pamyati Azieva were compared. The spike productivity traits of 100 spikes of wheatgrass, i.e., spike weight and length, number of spikelets and grains per spike, grain weight per spike, etc., were evaluated. The thousand kernel weight and spike harvest index were calculated. The grain yield and biomass were determined. The correlations of productivity components with plant height were analyzed. The grain morphometric parameters of variety Sova and spring bread wheat Pamyati Aziev (area, perimeter, length, width, and circularity) were compared. The grain and hay quality was evaluated. For wheatgrass (*Thinopyrum intermedium*), winter wheat variety Omskaya 4, and spring variety Element 22, the length, width, average diameter, volume, main area, number of root tips, and total root length were determined. The biological activity of the rhizosphere of *Thinopyrum intermedium* compared to winter wheat variety Omskaya 4, and spring bread wheat variety Element 22 was evaluated. The soil samples for accounting of microorganisms were taken in the shoots stage, after winter survival, and in the heading stage. It was found that grain yield, biomass, and hay of the variety Sova for three years of reproduction increased every year and averaged 9.2, 210.3, and 71.0 centner per hectare, respectively. Grain quality indicators were high, the 19.4 % protein and 36.3 % gluten content. The variety Sova has many grains per spike, on average more than 50, thousand kernel weight is 9.7 g, and

spike harvest index is 51 %. The length of all roots of *Thinopyrum intermedium* was 6.9-9.8 times longer compared to that of winter and spring varieties. The total number of agronomically important groups of microorganisms was 2.2 times higher, the intensity of mineralization (abundance of microorganism on starch ammonia agar to meat peptone agar SAA/MPA) was 58 % higher than in winter wheat variety Omskaya 4. The rate of cellulose decomposition was 13.7 and 21.4 % higher than in winter and spring wheat varieties. According to the studying the correlations between thousand kernel weight, plant height, and productivity traits suggest that the selection of biotypes with shorter stem, fewer spikelets and grains per spike is appropriate for grain weight increasing.

Keywords: *Thinopyrum intermedium*, spring wheat, winter wheat, breeding, perennial cultures, Sova variety, spike, valuable traits, correlations, yield, grain, hay, grain quality, roots, rhizosphere microorganisms

In recent decades, climate warming increased environmental threats and energy costs of grain production, wherefore perennial crops are proposed as an alternative to annual crops. The perennial crops are more resistant to adverse biotic and abiotic environmental factors, are able to preserve the soil cover, reduce the loss of soil moisture and nutrients, and absorb more carbon, reducing the greenhouse gases [1]. In general, in the world, greenhouse gas emissions from agriculture and land use account for more than 20% of their total amount in the atmosphere. In Russia, the Paris Climate Agreement was adopted by the Decree of the Government of the Russian Federation (September 21, 2019 No. 1228-PP), but has not yet been ratified. It is advisable to consider international practices for assessing and reducing the carbon footprint when developing the Russian system of crop production. The United States plans to radically reduce and completely eliminate pollution and greenhouse gas emissions in the agricultural sector by 2030.

In the world, annual grain crops provide about 70% of the energy consumed by humans with food, and occupy about 70% of the cultivated area [2]. An alternative strategy for use perennial crops involves domestication of wild species from the tribe *Triticeae* Dum. through multiple selections of plants with the desired agronomic traits.

In 2003, the Land Institute (Kansas, USA) launched a project to study perennial wild species. Among them, a gray wheatgrass [*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey] population was used to obtain a perennial cereal cultivar Kernza (six-cycle breeding for improved ear productivity, grain size and threshing). As a result, the grain yield increased by 77%, and the grain weight by 23% [3]. Programs for breeding perennial wheat through hybridization of cultivated wheat with perennial wild relatives were launched back in the 1930s in the USSR and the USA, but they still have not yielded the desired effect [4]. The results of these breeding programs contributed to the production of wheat forms with introgression of genes responsible for a long-term lifestyle, due to intergenomic or chromosomal translocations [5-7].

Progress has been achieved due to octaploid wheat-wheatgrass hybrids. A review on the state, achievements and prospects in using perennial wheat-wheatgrass hybrids ($2n = 56$) from the collection of Tsytsin Main Moscow Botanical Garden RAS) [8] showed the superiority of new forms in biological and economically useful traits compared to hybrids obtained in the USSR in 1927-1969. Stable selected genotypes have good performance in terms of overwintering over 2-3 years of life, grain yield, high grain protein, baking qualities, yield of green mass for three cuttings and resistance to diseases and pests. The cultivation of perennial crops is more eco-friendly and will reduce the cost for food and feed production [8].

Hybridization of wheat species with different wheatgrass species remains an effective method for introducing alien genetic variability into the common wheat genome [9-12]. Perennial wheats have been assessed in 20 countries [13]. Perennial life cycle in plants is under polygenic control, therefor, not a single

alien introgression but a combination of seven or more pairs of chromosomes transferred from a perennial donor into the cultivated wheat genome is necessary to provide the trait expression [14]. However, along with the perennial type of development, hybrid offspring possesses many undesirable traits characteristic of wheatgrass, such as spike fragility, difficult grain threshing, asynchronous ripening, and small grain size [15-17].

According to research data from the United States, identification of the cv. Kernza genes responsible for the domestication traits, primarily improved productivity, will increase the efficiency of breeding for wheatgrass domestication [3, 18]. Kernza has good grain quality, e.g., the grain protein level, lipids, fiber, and carotenoids are significantly higher than that of ordinary wheat [19-21]. It was found that for most of the intermediate wheatgrass (IWG) populations, grain antioxidant activity is higher compared to wheat [21]. Kernza® grain is used in bakery and confectionery production. Commercial products are currently marketed under the Kernza brand, owned by The Land Institute [1]. Kernza is widely used as a forage crop. In Canada, to increase its potential, crops of cv. Kernza mixed with legumes have been proposed as a long-term source of nitrogen instead of inorganic fertilizers [22].

In 2020, a unique large-grain wheatgrass cv. Sova of Omsk State Agrarian University was included in the State Register of Breeding Achievements Allowed for Use (State Register) for cultivation on all regions of Russia. The cv. Sova was created by mass selection of overwintered biotypes from the *Thinopyrum intermedium* population obtained from The Land Institute, followed by directed pollination and the creation of a new winter-hardy population

This work is the first to evaluate biological and economic characteristics of the large-grain wheatgrass cv. Sova from the State Register as an alternative to perennial wheat. The cv. Sova is recommended for cultivation for four to six years for grain and fodder. It forms high-protein grain and good quality hay.

Our aim was to evaluate the economically useful traits of a new large-grain wheatgrass cv. Sova in the conditions of the southern forest-steppe of Western Siberia and to reveal the correlation between the spike components and plant height to increase the efficiency of selection for improved thousand kernel weight.

Material and methods. During the field trials (55°02′N, 73°31′E, the Omsk State Agrarian University, the southern forest-steppe of Western Siberia, 2015-2019), the weather conditions varied and were typical for this geographic location; in general, moisture supply was insufficient, the hydrothermal coefficient (HTC) varied from 0.86 to 1.07. A new large-grain blue-gray wheatgrass cv. Sova, winter bread wheat (*Triticum aestivum* L.) cv. Omskaya 4, and spring bread wheat (*T. aestivum* L.) cv. Element 22 and cv. Pamyati Azieva were involved.

In experiment 1, the traits of spike and grain were studied in the cv. Sova (a breeding nursery, manual sowing on May 15, 2015; 5 m^2 plots, 40 cm row spacing, 10 cm plant spacing, sowing depth 2 cm, pure fallow predecessor, no replicates. In 2016-2017, the selection was carried out for the main spike at the stage of waxy ripeness. The ears were cut off to assess the yield components in 100 ears (i.e., ear weight and length, the number of spikelets and grains per ear, the number of grains per spikelet, grain weight per ear). The thousand kernel weight and the photosynthesis economic efficiency coefficient ($K_{econ.}$) were calculated. The hay quality was determined in 2015-2017 at the Omsk Agrochemical Center as per GOST R 55452-2013 (Moscow, 2014) in terms of the content of crude and digestible protein, fiber, feed units, metabolizable energy, calcium, phosphorus, carotene and sugars.

In experiment 2 (2017-2019), the grain yield and vegetative mass of cv. Sova were assessed in a nursery (manual sowing on August 15, 2016, 5 m^2 plots,

40 cm row spacing, 10 cm plant spacing, pure fallow predecessor, 3-fold replicates; manual harvesting). Sheaves were weighed. When the moisture content reached an air-dry state, sheaves were threshed (an MPSU-1 sheaf thresher, Omsk Experimental Plant, Russia) and the grain was weighed. Grain yields were adjusted to 14% moisture content. Also, the morphometric parameters of grain (area, perimeter, length, width, and circularity) were compared in the cv. Sova and spring bread wheat cv. Pamyati Aziev (an EPSON XL 110000 scanner, Seiko Epson Corporation, Japan; Smart grain v. 1.2 software (http://www.kazusa.or.jp/phenotyping/smartgrain/installation.html). The grain quality of the cv. Sova was determined using an Infralum FT 10 M device (Lumex, Russia).

In experiment 3, the correlation between the yield components and plant height was assessed in cv. Sova (a breeding nursery, sowing in May 2017, a SSFK-7 seeder, Omsk Experimental Plant, Russia; 0.2 ha sowing area, 40 cm row spacing, sowing depth 2 cm, the fallow predecessor, no replicates). In 2018-2019, 50 tall plants and 50 low-growing plants were collected to determine the main spike and the plant components.

In 2018-2019, cv. Sova plants were dug out to a shovel bayonet depth at flowering stage; the roots of winter wheat Omskaya 4 and spring wheat Element 22 were also dug out at flowering stage (5 plants of each cultivar, no repetitions). The roots were washed from the soil and their length, width, average diameter, volume, main area, the number of root tips, and total length were estimated using an EPSON XL110000 scanner (Epson America, Inc., USA) and WinRHIZO 2016 Pro software (Regent Instruments, Inc., Canada).

The winter and spring wheat cultivars were sown in the field where experiment 3 was located (the pure fallow predecessor, 500 seeds per 1 m^2, 3 repetitions). The winter wheat was sown on August 20 in 2017 and 2018, the spring wheat on May 20 in 2018 and 2019.

In experiment 4, the biological activity of the cv. Sova rhizosphere was compared to that of winter wheat Omskaya 4 and spring bread wheat Element 22 (the experimental field of the Omsk State Agrarian University, 2019). At tillering stage, after overwintering (May 22), and at earing stage (June 19), soil samples were taken to count rhizosphere microorganisms, the meat peptone agar (MPA) for saprophytic bacteria, including ammonifiers, starch-ammonia agar (SAA) for microorganisms that consume mineral nitrogen (NH_3), including actinomycetes, Czapek's medium for mushrooms, Mishustin's medium for oligonitrophils, Muromtsev-Gerretsen medium for phosphate mobilizing bacteria, Hutchinson's medium for cellulose-degrading microorganisms, leached agar with the addition of ammonium-magnesium salt of phosphoric acid for nitrifiers. The amount of nitrate nitrogen was determined by the disulfophenol method according to Grandval-Lyazh. To assess the soil biological activity, cellulose films were placed at a 0-20 cm depth in 4 replicates for each culture. The biological activity of the rhizosphere and soil was determined according to generally accepted methods [23-25] in a 3-fold repetition.

Statistical analysis included calculation of mean values (*M*), standard errors of means (±SEM), and correlation analysis. The differences were assessed by the least significant difference at a 5% significance level (LSD_{05}) according to the generally accepted methodology [26] using the package of applied statistical programs Microsoft Excel. Two-way analysis of variance was performed using the STATISTICA v. 6.0 (StatSoft, Inc., USA).

Results. Ear productivity is one of the main elements of the crop structure. In the cv. Sova, the thousand kernel weight averaged 9.7 g. It should be noted that cv. Sova had higher grain number per spike and $K_{econ.}$ of ears (Table 1).

1. Yield components in *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova (*M*±SEM; experiment 1, the field of the Omsk State Agrarian University)

Trait	2016	2017	Average over two years
Ear weight, g	0.79±0.19	0.97±0.23	0.88±0.21
Ear length, cm	18.2±2.3	19.8±2.5	19.0±2.4
The number of spikelets per ear	18.3±2.7	21.0±3.1	19.6±2.9
The number of grains per ear	41.3±13.3	60.0±19.2	50.6±16.5
The number of grains per spikelet	2.3±0.7	2.9±0.9	2.6±0.8
Ear grain weight, g	0.4±0.1	0.5±0.1	0.4±0.1
Thousand kernel weight, g	10.4±1.5	9.0±1.3	9.7±1.4
Ear $K_{econ.}$, %	50.6±7.0	51.5±8.0	51.0±7.5

N o t e. $K_{econ.}$ — photosynthesis economic efficiency coefficient. For a description of the experiment, see the *Material and methods* section.

The breeding improvement of *Th. intermedium* largely depends on the efficiency of selection based on the contingency of plant traits. We found a medium relationship of the thousand kernel weight with the stem height and the ear length (r = 0.3; p = 0.05) (Fig. 1). A negative correlation occurred between the thousand kernel weigh and the spikelet number (r = –0.5, p = 0.01) and also the grains number per ear (r = –0.5; p = 0.01). Probably, the selection of shorter-stemmed clones, especially those with fewer spikelets in the main spike and, accordingly, fewer grains, is advisable to further increase the thousand kernel weigh.

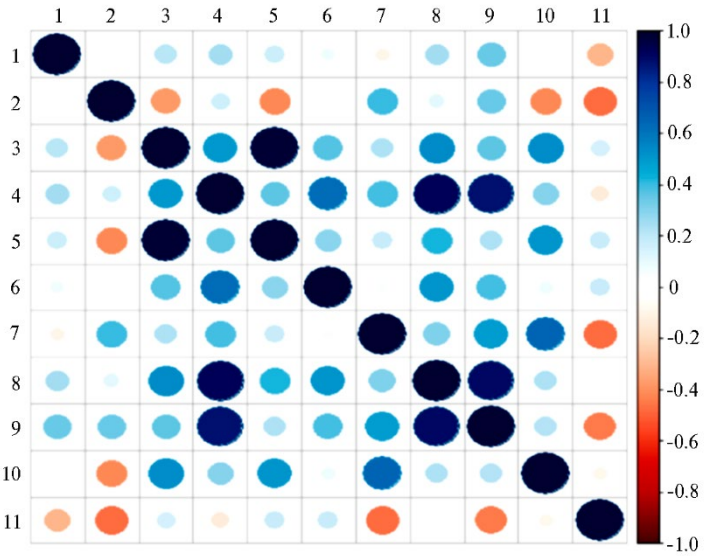

Fig. 1. Correlation (r) between traits of *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova: 1 — plant height, 2 — ear length, 3 — plant weigh, 4 — ear weight, 5 — stem weight, 6 — ear width, 7 — spikelet number per ear, 8 — grain weigh per ear, 9 — grain number per ear, 10 — ear density, 11 — thousand kernel weight. The critical r values at p = 0.05 and p = 0.01 are 0.27 and 0.39, respectively (experiment 3, the field of the Omsk State Agrarian University, 2018-2019). For a description of the experiment, see the *Material and methods* section.

2. Grain traits in *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova and spring bread wheat *Triticum aestivum* L. cv. Pamyati Azieva (*M*±SEM; experiment 2, the field of the Omsk State Agrarian University)

Parameter	cv. Sova			cv. Pamyati Azieva			LSD_{05}
	2017	2018	2019	2017	2018	2019	
Area, mm^2	9.28±1.45	9.25±1.45	9.03±1.41	15.98±2.50	15.11±2.36	14.48±2.26	1.1
Perimeter, mm	16.34±1.67	16.87±1.72	15.91±1.63	16.82±1.72	15.83±1.62	14.23±1.46	1.3
Length, mm	7.02±0.67	7.35±0.71	6.83±0.66	6.47±0.62	6.21±0.59	6.45±0.62	0.55
Circularity	0.44±0.05	0.46±0.05	0.46±0.05	0.72±0.08	0.72±0.08	0.70±0.07	0.25
Width, mm	1.68±0.25	1.72±0.25	1.67±0.24	3.29±0.48	3.30±0.48	3.00±0.44	0.81

N o t e. For a description of the experiment, see the *Material and methods* section.

The wheatgrass cv. Sova has a long, but small, incomplete grain, as evidenced by the reduced area and circularity compared to the spring bread wheat cv. Pamyati Azieva (Table 2, Fig. 2).

Fig. 2. Grain of the spring bread wheat *Triticum aestivum* L. cv. Pamyati Azieva (A) **and wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova** (B) (the field of the Omsk State Agrarian University).

The cv. Sova is a dual-purpose crop for grain and also for green fodder and hay production. Its grain, green mass and hay yields increased over 3 years (Table 3). The grain protein and gluten levels in the new variety turned out to be very high. An interesting fact is that in the third year of reproduction, the grain protein was 2% higher. The wet gluten content was the highest in 2018 (see Table 3).

3. Yields and quality traits in wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova (*M*±SEM; experiment 2, the field of the Omsk State Agrarian University)

Year	Yield, c/ha			Protein, %	Gluten, %
	grain	green mass	hay		
2017	8.3±2.1	187.1±48.8	63.0±15.3	18.5±2.0	35.6±4.1
2018	9.0±2.3	219.0±53.2	73.0±18.6	19.1±2.4	37.2±4.3
2019	10.4±2.7	224.8±54.3	76.4±18.8	20.5±2.2	36.0±4.4
Average	9.2±2.4	210.3±51.2	71.0±17.8	19.4±2.3	36.3±4.2
LSD_{05}	1.2	13.8	5.0	1.3	1.1

N o t e. For a description of the experiment, see the *Material and methods* section.

The hay quality was quite suitable for cattle feeding. The quality parameters were the best in 2015 in experiment 1 when the plants were harvested only for hay, i.e., prior to earing. In 2016-2017, the hay quality was assessed in experiment 1 after the grain was threshed (Table 4).

The perennial life cycle and well-developed root system of the wheatgrass plants significantly improve their ability to utilize soil moisture reserves. In the upper soil layer, the root system of the cv. Sova was more developed compared to the wheat plants (Table 5). In 2018, given maximum possible width and length of the roots 25×25 cm limited by the size of soil cube with roots, the root size was 23.4×24.7 cm in the wheatgrass vs. 8.0×11.6 cm in winter wheat cv. Omskaya 4 and 7.2×10.7 cm in spring wheat cv. Element 22. The average area of the main roots in the wheatgrass was larger than that of winter (7.9-fold, $p \leq 0.05$) and spring (8.4-fold, $p \leq 0.05$) wheats, and the area of all roots was 8.0 times larger ($p \leq 0.05$) and 8.4 times larger ($p \leq 0.05$), respectively, the roots were 0.3 and 0.1 mm larger in diameter, the root volume was 7.8 times and 6.2 times larger ($p \leq 0.05$), the length of all roots was 6.9 and 9.8 times ($p \leq 0.05$) greater, the average number of root tips was 3.7 and 4.6 times greater (see Table 5). In 2019, similar differences between the cultures persisted. Consequently, the wheatgrass cv. Sova is significantly superior to both tested spring and winter wheat cultivars in all traits of the root system.

4. Hay quality parameters in wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova (*M*±SEM; experiment 1, the field of the Omsk State Agrarian University)

Year	Chemical composition, %		Feed units, dry matter	Metabolizable energy, MJ/kg	Digestible protein, g/kg	Ca, g/kg	P, g/kg	Carotene, mg/kg	Sugars, g/kg
	protein	fibers							
2015	14.30±2.11	21.50±1.96	0.55±0.04	8.55±0.51	95.7±20.3	3.08±0.43	2.20±0.31	53.00±6.11	215.40±3.41
2016	5.93±0.72	31.50±3.21	0.49±0.03	7.62±0.47	39.7±9.3	3.83±0.52	1.64±0.26	18.00±3.24	38.30±3.93
2017	9.39±1.56	28.10±3.02	0.52±0.03	8.44±0.52	62.9±14.2	4.32±0.63	1.78±0.27	28.00±4.49	70.40±6.78
LSD_{05}	6.42	8.57	0.08	0.96	33.3	1.05	0.53	24.0	131.1

N o t e. For a description of the experiment, see the *Material and methods* section.

5. Root traits in wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova, winter bread wheat *Triticum aestivum* L. cv. Omskaya 4 and spring bread wheat *T. aestivum* L. cv. Element 22 (*M*±SEM; experiment 3, the field of the Omsk State Agrarian University)

Trait	cv. Sova		cv. Omskaya 4		cv. Element 22		LSD_{05}
	2018	2019	2018	2019	2018	2019	
Root width, cm	23.4±2.3	25.1±0.7	8.0±1.0	8.6±1.2	7.2±0.7	8.6±0.7	8.79
Root length, cm	24.7±2.2	26.2±0.6	11.6±1.5	12.2±1.5	10.7±0.9	11.6±0.8	7.58
Main root area, cm^2	58.3±9.0	57.9±7.2	7.4±2.3	7.6±1.9	6.9±2.1	6.1±1.5	27.70
Total area, cm^2	183.2±28.4	181.9±22.7	23.1±7.1	22.8±6.8	21.7±4.7	19.2±4.7	87.18
Average diamter, mm	0.90±0.10	0.70±0.05	0.06±0.09	0.58±0.08	0.80±0.06	0.60±0.07	0.31
Root volume, cm^3	3.10±0.70	3.21±0.70	0.40±0.09	0.41±0.09	0.50±0.09	0.30±0.10	1.49
Total root length, cm	854.2±42.5	826.3±56.9	124.3±25.5	138.5±30.3	87.4±20.7	96.5±19.0	395.4
Root tip number	1643.0±460.0	2099.0±261.0	439.9±129.9	450.3±131.2	355.0±79.2	360.9±76.3	811.6

N o t e. For a description of the experiment, see the *Material and methods* section.

6. Rhizosphere microbial community composition (CFU/g) **in wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova and winter bread wheat *Triticum aestivum* L. cv. Omskaya 4** (*M*±SEM; experiment 4, the field of the Omsk State Agrarian University, 2019)

Microodganisms	cv. Omskaya 4			cv. Element 22			Average value for cv. Sova vs. cv. Omskaya 4, %	LSD_{05}
	dated		average	dated		average		
	05/22	06/19		05/22	06/19			
Bacteria on MPA, $\times 10^6$	25.0	19.4	22.2±1.8	31.0	26.1	28.6±1.5	+28.8	6.3
Microorganisms on SAA, $\times 10^6$	15.1	14.3	14.7±0.3	42.0	22.5	32.3±6.2	+119.7	6.6
Oligonitrophylic bacteria, $\times 10^6$	77.0	69.0	73.0±2.5	328.2	46.3	187.3±89.1	+156.5	96.0
Phosphate mobilizing bacteria, $\times 10^6$	84.0	51.1	67.6±10.4	209.8	51.6	130.7±50.0	+93.3	44.1
Fungi, $\times 10^3$	38.0	28.5	33.3±3.0	52.8	50.0	51.4±0.9	+54.4	42.5
Cellulosolytics, $\times 10^3$	70.6	27.0	48.8±13.7	87.9	31.7	59.8±17.7	+22.5	42.6
Nitrifying bacteria, cells	163.0	220.3	191.7±18.0	131.0	288.6	210.0±49.8	+9.5	161.0
Total, $\times 10^6$	201.4	154.1	177.8±14.0	611.3	146.9	379.2±146.8	+281.4	147.0

N o t e. MPA — meat peptone agar, SAA — starch ammoniac agar. For a description of the experiment, see the *Material and methods* section.

The total number of groups of agronomically useful microorganisms in the rhizosphere of the wheatgrass cv. Sova exceeded that in winter wheat Omskaya 4 by 201.4×10^6 CFU/g, or 2.1 times ($p \le 0.05$). However, an increase in microbial abundance varied for each of the identified groups, being the greatest in oligonitrophylic and phosphates mobilizing bacteria (by 114.3×10^6 and 63.1×10^6 CFU/g, respectively). The number of these microbes particularly significantly increased (up to 328×10^6 and 210×10^6 CFU/g) during tillering of the wheatgrass in May when the soil moisture was still enough (more than 20%). Oligonitrophylic bacteria can survive under a lack of soil nitrogen and fix atmospheric nitrogen [27]. Phosphate-mobilizing microorganisms isolated on the Muromtsev-Gerretsen medium transform mineral phosphorus compounds that are difficult to access for plants in the soil, which improves phosphorus nutrition of plants. It can be assumed that in the wheatgrass of the third year of reproduction the root excrete composition is more favorable for the growth and vital activity of these microorganisms than in winter wheat (Table 6).

According to Sindireva et al. [28], the numerical ratio of different types of microorganisms serves as a better indicator of conditions than the number of one species, since the whole better reflects the total impact than the part. The ratio of groups of microorganisms on SAA to MPA assesses the intensity of mineralization of soil organic nitrogen-containing compounds while that on MPA to SAA indicates the intensity of nitrogen immobilization [29]. On average, according to preliminary data, mineralization in the rhizosphere of wheatgrass was more intense (SAA/MPA = 1.1), while immobilization of nitrogen and its accumulation by organic soil compounds prevailed in the rhizosphere of winter wheat plants, as SAA/MPA = 0.66 evidenced. The abundance of soil fungi and cellulose-decomposing microorganisms in the rhizosphere of wheatgrass was also higher than under winter wheat, by 11×10^3 and 18×10^3 CFU/g, or by 22.5 and 54.4%.

Stimulation of soil microflora in the rhizosphere of wheatgrass is apparently associated with the peculiarities of the root system of this perennial cereal crop. Thus, the total root area in wheatgrass exceeded that in winter wheat almost 8.0-fold, and the root width 2.9-fold (see Table 5). Cellulose test, according to the author of the method Tikhomirova [30], characterizes in general the effective fertility under the crop. We observed the highest activity of soil microflora under wheatgrass compared to winter and spring wheats, as evidenced by the intensity of cellulose decomposition in the 0-20 cm layer, 57.0, 43.3, and 35.6% (Table 7).

7. Cellulose decomposition (%) in soil under wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey cv. Sova, winter bread wheat *Triticum aestivum* L. cv. Omskaya 4 and spring bread wheat *T. aestivum* L. cv. Element 22 ($M\pm$SEM; experiment 4, the field of the Omsk State Agrarian University, 2019)

Crop (A)	Soil layer (B)			
	0-10 см	10-20 см	0-20 см	min-max
Wheat cv. Omskaya 4	49.8±4.9	36.7±5.2	43.3±4.1	33.8-59.1
Wheatgrass cv. Sova	60.9±2.9	53.2±6.6	57.0±2.4	47.2-66.2
Wheat cv. Element 22	34.0±1.2	37.2±4.8	35.6±1.0	31.5-41.4

Note. LSD_{05}: A = 11.0, for B = 6.9, AB = 15.5. For a description of the experiment, see the *Material and methods* section.

In addition to traditional breeding methods, the cultivation of wild wheat relatives, such as *Thinopyrum intermedium*, is undoubtedly promising for agriculture which should be regenerative and resistant to climate change. Back in the 1980s, Jackson [31] suggested that the maximum sustainable crop yields can be obtained through the use of perennial crops. Based on the findings of The Land Institute, and additional breeding for winter hardiness, followed by targeted pollination of the isolated clones, we have created the Sova cultivar adapted for

Russia conditions. For Russian agriculture, the cv. Sova is the first large-grain wheatgrass crop with a thousand kernel weight of 9.0-10.4 g (see Table 1). Despite the fact that the cv. Sova is significantly inferior to the spring and winter wheats in thousand kernel weight, its grain can be used in bakery and confectionery products, as evidenced by the example of a similar wheatgrass cv. Kernza in the USA [1]. A further increase in the thousand kernel weight is among the main challenges of the wheatgrass breeding. Given the correlations between the spike traits (see Fig. 1), the selection of forms with a smaller number of spikelets and a smaller number of grains per main spike seems advisable to further increase the thousand kernel weight. The medium negative correlation between the plant height and the thousand kernel weight ($r = -0.3$; $p = 0.05$) is positive in breeding forms for grain use, since taller populations are more prone to lodging.

In the works of The Land Institute, in the fourth breeding cycle, the kernel weigh had a high heritability in the wheatgrass population ($h^2 = 0.68$), but this trait was studied in plants with the largest grain weight per ear [32]. The length of the grain in the cv. Sova cultivar corresponded to that of common wheat, however, the circularity, indicating the filling of the grain, was significantly less than in the wheat cv. Pamyati Aziev (see Table 2). Breeding for an increased circularity which is closely related to the grain weight per volume is of certain value in wheatgrass breeding for grain use. The grain yield of perennial cereals is lower than that of annual crops, because part of the energy is spent on the development of the root system and branching after overwintering. However, since the cost of grain production is lower, you can get a net profit, despite the low yield of wheatgrass compared to annual cereals, as evidenced by the experience of American researchers [33]. It is possible to cultivate wheatgrass without loss for four to six years, while the appropriate agricultural technology and mineral fertilizing in the spring will increase the crop yield.

The grain protein content in the cv. Sova cultivar was high (see Table 3). The amount of protein in the wheatgrass grain is significant, but the content of high molecular weight glutenin subunits which determine the high baking quality is less than that of bread wheat. A higher total content of dietary fiber in wheatgrass flour (16.4%) than in whole wheat flour (11.0%) is also indicated [34]. Dietary fibers in a person's diet are helpful in controlling blood glucose after a meal. Coarse wheatgrass flour contains a significant amount of dietary fiber and antioxidants [19, 34]. It was found that for most of the wheatgrass populations, a higher antioxidant activity in grain is characteristic as compared to wheat [21].

Increasing erosion is the most common soil degradation process worldwide. Soil erosion leads to a decrease in soil fertility, pollution of receiving water bodies downstream and an increase in greenhouse gas emissions [35, 36]. The roots of the cv. Sova is significantly superior to that of spring and winter wheats, which favorably affects the biological role of this variety in increasing fertility and reducing negative erosion processes in the soil (see Table 5)

Microorganisms play an important role in the main soil processes that determine the level of root nutrition in plants. The intensity and direction of microbiological processes contribute to the better utilization of mineral and organic compounds [37]. Species and varieties have their specific root excretes which contain organic physiologically active compounds — enzymes, vitamins, growth substances, etc. [38]. Therefore, in the root zone, abundant saprophytic microflora multiplies in an abundance more significant than in the soil mass. Our experiments showed that the conditions in the rhizosphere of wheatgrass cv. Sova are more favorable for the development of agronomically important microorganisms a compared to the winter wheat cv. Omskaya 4. Their total number exceeded 2.2 times

the corresponding values in the winter crop. The mineralization (SAA/MPA) was 58% higher, the cellulose decomposition 13.7 and 21.4% higher, respectively, than in the varieties of winter and spring wheat (see Tables 6, 7). These results are consistent with reports from other research groups [39-41].

To summarize, it should be noted that the wheatgrass breeding for economically useful traits is promising to realize the genetic potential of the crop. The expansion of the cultivation areas of perennial crops will contribute to the rational use of natural resources, improve the phyto climate, balance the diet of the population and, in general, will contribute to the ecological improvement, the accumulation of carbon dioxide in the soil and the reduction of negative greenhouse effects. At present, the main breeding technique for wheatgrass is selection for economically useful traits. However, in the short term, the efficiency of marker-assisted selection (MAS) may become significantly higher. We expect that breeding progress will be accelerated through MAS programs. Their use together with new breeding tools will improve the wheatgrass yield and agronomic traits.

Thus, the grain, green mass and hay yields of the perennial wheatgrass cv. Sova for three years of reproduction increased every year and averaged 9.2, 210.3, and 71.0 c/ha, respectively. The grain quality parameters of the new variety were high (19.4% protein and 36.3% gluten). The grain number per ear was on average more than 50 pieces, the 1000 grain weight was 9.7 g, $K_{econ.}$ of ears 51%. The roots of the cv. Sova variety is 6.9-9.8 times longer, and the total surface area of roots is 8.0 times larger than that of winter and spring bread wheats. The total abundance agronomically useful groups of microorganisms in the rhizosphere of the cv. Sova turned out to be 2.2 times higher, with 58% higher intensity of mineralization (by the ratio of microorganisms grown on SAA to MPA) as compared to the winter wheat cv. Omskaya 4. Cellulose decomposition was 13.7 and 21.4% higher than that of winter and spring wheat varieties. The study of correlations between the thousand kernel weight, plant height, and productivity traits revealed the expediency of selecting shorter-stemmed biotypes with a smaller spikelet number and grain number per main spike in order to further increase the grain size. The cv. Sova variety is included in the State Register of Breeding Achievements Permitted for Use and is recommended for cultivation in the regions of Russia for four to six years for fodder and grain purposes.

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PRODUCTION AND ANALYSIS OF COMPOSITE TOMATO PLANTS *Solanum lycopersicum* L. CARRYING PEA GENES ENCODING THE RECEPTORS TO RHIZOBIAL SIGNAL MOLECULES

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Abstract

The development of legume-rhizobial symbiosis is based on signal exchange between partners, which ensures their mutual recognition and activation of the infection process and the program of nodule organogenesis. In this regard, it is of great interest to study the possibility of acquisition by non-legume plants of the ability to perceive lipochito-oligosaccharide signal molecules of rhizobia, the Nod factors, and subsequent activation of signal transduction pathway. To study this possibility in our work, we carried out the transfer of the genes encoding receptors to Nod factors of legume plant pea *Pisum sativum* L. into tomato *Solanum lycopersicum* L. (Carmello cultivar) using the transformation with *Agrobacterium rhizogenes*. In pea, two receptor kinases, SYM10 and K1, were previously identified, which are necessary for the recognition of Nod factors during the initiation of symbiosis with rhizobia. Upon reception of Nod factors, a complex is formed between these two receptor kinases, which leads to signal transduction. In the present work, we carried out the transfer of two genes encoding LysM-RLK SYM10 and K1 in pea *P. sativum* into tomato plants *S. lycopersicum* using agrobacterial transformation. In composite plants transformed with *PsSym10* or *PsK1* genes, the possibility of expression activation of introduced receptor genes in response to inoculation with a typical rhizobial strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 was shown. It was also shown that, under the influence of receptors in genetically transformed roots of composite plants, the expression of genes is increased, which can be regulated by components of the "common" signal pathway. The aim of this work was to study the possibility of acquiring the ability of *S. lycopersicum* plants to recognize signal molecules of rhizobia after transfer of the genes encoding receptors for Nod factors in the legume plant *P. sativum*. Two types of constructs in the pKm43GW vector were obtained and used, in which the *PsSym10* or *PsK1* genes encoding receptors were cloned under the pSlEXT1 promotor of tomato extensin gene — pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S. Young tomato seedlings of *S. lycopersicum* cv. Carmello were transformed with the *Agrobacterium rhizogenes* Arqua 1 strain. The transformed seedlings were placed on Murashige-Skoog (MS) agar medium without sucrose in Petri dishes and cultured in an upright position in a phytotron until callus is appeared. After that, the plants were transferred to MS medium with 3 % sucrose containing 0.3 mg/ml of the antibiotic cefotaxime and incubated until transgenic roots are appeared. Composite plants were transferred into vermiculite poured with 0.5× Fahreus medium and incubated under high humidity conditions for 2-3 days. The plants were then inoculated with *R. leguminosarum* bv. *viciae* CIAM1026 containing the *uidA* glucuronidase gene (*GUS*). For the analysis we used transformed roots of composite tomato plants without rhizobial inoculation (control, 7 days), as well as transformed roots at 7 and 21 days after inoculation. The analysis of gene expression was performed by quantitative PCR combined with reverse transcription (RT-PCR). In genetically transformed roots of tomato plants the expression of both *PsSym10* and *PsK1* genes was observed under the pSlEXT1 promoter, moreover the expression was enhanced under the influence of rhizobial inoculation. A significant (approximately 2.0-2.5-fold) increase in the expression of the *PsSym10* gene was shown in response to inoculation with rhizobia both at 7 and 21 days. The level of *PsK1* expression was found to be the highest 7 days after inoculation in the transformed roots of composite tomato plants as compared to the control. To determine whether the components of the "common" signal pathway will be activated under the influence of transferred receptors in composite tomato plants, the changes in the expression of *S. lycopersicum SlD27*, *SlNSP2*, *SlRAM1*,

and *SlMAPK6* genes were assessed. These genes encode carotenoid isomerase (DWARF27) which regulates the synthesis of the hormones strigolactones, transcription factors NSP2 and RAM1, and mitogen-activated protein kinase (MAPK6). Activation of the expression of two genes, the *SlNSP2* and *SlMAPK6* in response to inoculation may indicate the effect of the introduced pea *K1* gene on the susceptibility of tomato plants to rhizobial inoculation.

Keywords: legume-rhizobial symbiosis, receptor-like kinases, Nod factors, composite plants, gene expression.

The ability for symbioses with *Rhizobiales* nitrogen-fixing bacteria called rhizobia is evolutionarily beneficial and gives plants an advantage with a lack of soil nitrogen. However, the plants involved in symbiotic interactions are restricted to the order *Fabales* and some members of the *Rosales*, namely several *Parasponia* species of the *Cannabaceae* family [1]. New host plants capable of entering symbiosis with rhizobia can be prospective for efficient farming with minimum application of nitrogen fertilizers. A crucial step is to assess the ability of non-leguminous plants' receptors to percept rhizobial signaling molecules and to transmit molecular signals which allow the symbiotic partners to recognize each other.

Lipochito-oligosaccharide signaling molecules (Nod-factors) secreted by rhizobia serve as key mediators of symbiosis in leguminous plants. The organogenesis of nodules and bacterial colonization depend on the recognition of these molecules by the host plant [2]. Compounds of a very similar structure — lipochito-oligosaccharides (Myc-factors) secreted by fungi of arbuscular mycorrhiza (AM) [3], play the role of signaling molecules necessary for the development of another type of symbiosis with AM fungi formed by more than 80% of terrestrial plants. Activation of responses upon recognition of Nod factors may be associated with the presence of signaling pathway components in non-leguminous plants that are common for signal transmission during the development of legume-rhizobial symbiosis and symbiosis of plants with AM fungi [4, 5]. The differences are mainly in the recognition of Nod and Myc factors by different receptors and the activation of transcription factors specific for each pathway which stimulate their target genes [6, 7].

The LysM receptor-like kinases (LysM-RLK family) with specific lysine motifs (LysM) in the extracellular domains are involved in binding Nod and Myc factors. Among LysM- RLK, it is customary to discriminate LYK (LysM-receptor-like kinases) proteins with an active kinase domain and LYR (LYK related) proteins with inactive kinase domain. The presence of LysM motifs determines the ability to bind compounds containing N-acetylglucosamine residues (Nod and Myc factors consist on average of 4-5 N-acetylglucosamine residues and contain a specific fatty acid at the non-reducing end of the molecule) [8-10]. Some members of the LysM-RLK family recognize structurally similar molecules containing N-acetylglucosamine residues, such as chitin, peptidoglycan murein, and their low molecular weight derivatives [11, 12]. It turned out that upon reception of both Nod and Myc factors, a complex should be formed between LYR and LYK proteins to generate the signal transduction in plants [13, 14].

Some non-leguminous plants can also enter symbiosis with nitrogen-fixing rhizobia. LysM-RLK PanNFP, a kinase of LYR class found in *Parasponia andersonii* Planch. (*Cannabaceae*), recognizes signals from both rhizobia and AM fungi [15]. It is assumed that PanNFP forms complexes with different co-receptors (LYK) upon recognition of Nod and Myc factors. LysM-RLK SlLYK10 (LYR), a kinase recently identified in tomato *Solanum lycopersicum* L., shows high homology to the Nod factor receptor of leguminous plants — MtNFP of alfalfa *Medicago truncatula* Gaertn. [16]. The SlLYK10—co-receptor LysM-RLK SlLYK12 (LYK) complex binds Myc factors. Moreover, the introduction of the *SlLYK10* gene under a strong promoter into the *M. truncatula nfp* mutants defective in the

Nod factor receptor gene restores nodulation and the formation of functional nodules [17, 18]. The Nod factor recognition restored in leguminous plants due to the expression of tomato receptor gene explains the responsiveness of the tomato cell culture to exogenous Nod factors and development of the earliest responses to these signals, i.e., alkalization of the growth medium and depolarization of the membrane [19]. It can be assumed that the recognition of Nod factors in tomato plants is associated with the activation of the SlLYK10/SlLYK12 Myc factor receptor complex due to the similarity of the structure of signaling molecules. Indeed, a recent study of LysM-RLK SlLYK10 showed that this receptor binds not only Myc factors but also Nod factors with high affinity [18]. Such susceptibility of non-leguminous plants to signaling molecules of rhizobia suggests that these molecules can activate the components of "common" signaling pathway (CSP). Therefore, it is of interest to introduce genes encoding rhizobial Nod factor receptors highly affine and specific to the rhizobial signaling molecules into non-leguminous plants and to assess the plant response to the rhizobial signals. In particular, it is necessary to find out i) whether the components of the CSP are involved in the signal transduction and ii) what transcription factors and target genes are activated.

In the pea *Pisum sativum* L., two LysM-RLKs, SYM10 and K1 (LYR and LYK), were identified, which are necessary for the recognition of Nod factors during the initiation of symbiosis with rhizobia [20, 21]. Note, pea mutants for the *sym10* and *k1* genes almost completely lack the ability to respond to inoculation with rhizobia and exogenous Nod factors that indicates the important role of these receptors for symbiosis [20, 21].

Here, two *P. sativum* genes encoding LysM-RLK SYM10 and K1 were transferred to tomato plants *S. lycopersicum* using Agrobacterium-mediated plant transformation. It was revealed for the first time that inoculation of the composite plants with a typical rhizobium strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 activates the expression of *PsSym10* or *PsK1* genes. In genetically transformed roots of the composite plants, the expression of genes that CSP components can regulate also increases due to the receptors.

We transferred *Pisum sativum* genes for Nod factor receptors to *Solanum lycopersicum* to find out if tomato plants will acquire the ability to recognize rhizobial signaling molecules.

Materials and methods. Seeds of tomato (*Solanum lycopersicum*) cv. Carmello were sterilized with 15% NaOCl (0.1 M) for 5 min, washed 6-fold with distilled sterile water, exposed to 10% H_2O_2 for 2 min, and washed 3-fold with large volume of sterile water. Sterilized seeds were placed in Petri dishes on agarized Murashige-Skoog (MS) medium without sucrose [22] and incubated in the dark for 1 day at 4 °C followed by incubation in the dark for 5-7 days at room temperature for germination. Young seedlings were transferred to sterile 400 ml pots with MS medium supplemented with 3% sucrose and grown in a MLR-352H phytotron (Panasonic, Japan) at 21 °C, 60% humidity and a 16 h light/8 h dark regime.

The *Rhizobium leguminosarum* biovar *viciae* CIAM1026 strain was cultured at 28 °C on tryptone yeast agar (TY) with 0.5 mg/ml streptomycin. *Escherichia coli* XLBlue MRF' and TOP10 strains (Thermo Fisher Scientific, USA) were used for standard cloning procedures. The *Agrobacterium rhizogenes* Arqua 1 strain, containing the required construct, was used to obtain composite plants. *A. rhizogenes* Arqua 1 was cultured at 28 °C on TY agar.

Two constructs, the pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S were generated for plant transformation. The coding full-length

sequence of the *PsSym10* gene lacking a stop codon was amplified using cDNA as a template (total RNA was isolated from nodules of Finale peas collected on day 21 after inoculation) [20]. Amplification was performed using high-precision Phusion polymerase (Thermo Fisher Scientific, USA) [20]. The *PsSym10* gene sequence was fused with the 3xFLAG coding sequence and transferred into the pDONR™ 221 vector (Thermo Fisher Scientific, USA). We also used previously designed construct containing the full-length coding sequence of the *PsK1* gene (without a stop codon) fused with the sequence encoding the RFP fluorescent protein cloned in the pDONR™ 221 vector [20].

pDONR L4-pEXT1-R1r vector containing the pEXT1 tomato extensin gene promoter (1121 bp) was provided by Dr. S. Bensmihen (Institut National de la Recherche Agronomique, Toulouse, France). The pDONR L4-pEXT1-R1r was used for multilocus homologous recombination. The pDONR™ 221 vectors containing the coding sequences of the *PsK1* gene or the PsSym10 gene and the pENTRY R2-T35S-L3 vector with the T35S terminator (Ghent University, Belgium) were also used for recombination. The constructs were cloned into the pKm43GW delivery vector (pDEST4-3) using LR-clonase II (Thermo Fisher Scientific, USA). pKm43GW was used as the final vector for homologous recombination cloning. The resulting constructs in the pKm43GW vector (pKm43GW-pSlEXT1::*PsSym10-3xFLAG*::T35S and pKm43GW-pSlEXT1::*PsK1-RFP*::T35S) were electroporatically transferred into *A. rhizogenes* Arqua 1 strain.

For transformation, young tomato seedlings cut off at the hypocotyl were treated with *A. rhizogenes* Arqua 1 suspension. Four or five transformed seedlings were placed on MS agar medium without sucrose [22] in a Petri dish between two sheets of filter paper soaked in sterile distilled water. The root area was covered with foil. The dishes were placed in the phytotron and cultured upright for 10-14 days at 21 °C, 60% humidity, and 16 h light/8 h dark until the appearance of callus. Then the plants were incubated under the same conditions for 5-10 days on the MS medium with 3% sucrose and 0.3 mg/ml cefotaxim until the appearance of transgenic roots. The composite plants covered with transparent plastic bags were grown at high humidity, 21 °C, and 16 h light/8 h darkness on vermiculite poured with 0.5× Fahreus medium (0.132 g/l $CaCl_2$, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l KH_2PO_4, 0.075 g/l $Na_2HPO_4 \cdot 2H_2O$, 5 mg/l Fe-citrate, 0.07 mg/l $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, H_3BO_3 and $Na_2MoO_4 \cdot 2H_2O$, pH 7.5). In 2-3 days, the plants were inoculated with *R. leguminosarum* bv. *viciae* CIAM1026 carrying the *uidA* glucuronidase gene (*GUS*) (2 ml of OD_{600} = 0.5 suspension per plant). After cultivation for 7 and 21 days, total RNA was isolated from the transformed roots carrying the pSlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S construct to synthesize cDNA. Transgenic plant roots without rhizobia inoculation were used as a control.

In all experiments, total RNA was extracted using the PureZOL™ RNA Isolation Reagent (Bio-Rad Laboratories, USA) according to the manufacturer's protocol. DNAseI treatment (Thermo Fisher Scientific, USA) was applied to remove genomic DNA. cDNA was synthesized using reverse transcriptase RevertAid H Minus (Thermo Fisher Scientific, USA) with oligo(dT18) primer (Sileks, Russia) as per the manufacturer's protocol.

Gene expression was analyzed by quantitative reverse transcription PCR (RT-PCR) (a CFX96 Real-Time System amplifier, Bio-Rad Laboratories, USA) with iQ SYBR Green Super Mix (Bio-Rad Laboratories, USA). RT-PCR conditions: 30 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (40 cycles). All primers were designed using the DNAStar program and synthesized by the Evrogen company (Russia, http://www.evrogen.com).

To calculate the averaged values of the relative gene expression and the

standard error of the mean (±SEM), the built-in functions of Microsoft Excel were used.

Results. We used the *Agrobacterium rhizogenes* Arqua 1 strain containing the pSlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S constructs to transform tomato plants of cv. Carmello with pea genes *PsSym10* and *PsK1* for receptors of Nod factors.

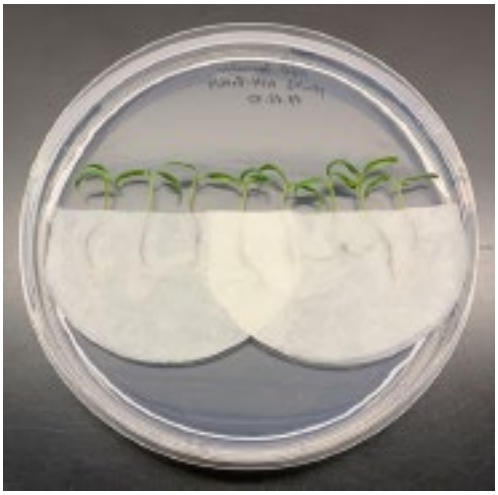

Fig. 1. Agrobacterium-mediated transformation of tomato (*Solanum lycopersicum* L., cv. Carmello).

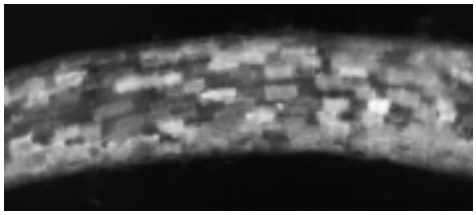

Рис. 2. Root of a composite tomato plant (*Solanum lycopersicum* L., cv. Carmello) transformed with the pSlEXT1::*PsK1-RFP*::T35S construct (a fluorescent binocular microscope. Zeiss, Germany; luminescence of the fluorescent protein RFP in cells).

We reveal a selective luminescence of genetically transformed roots with the pSlEXT1::*PsK1-RFP*::T35S construct in which the gene of K1 receptor was fused with the sequence encoding the fluorescent protein RFP (Fig. 2). This indicated the expression of the transferred gene in the transformed tomato roots. We transformed the plants with genes encoding full-length protein sequences with N-terminal signal peptides. The degree of RFP luminescence confirmed normal function of the signal peptides.

The next step was to assess the expression of the *PsSym10* and *PsK1* genes in the roots of composite tomato plants possessing SlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S constructs upon inoculation with *R. leguminosarum* bv. *viciae* CIAM10267 (in 7 and 21 days) and without inoculation (in 7 days, the control). The table shows sequences of the primers in the RT-qPCR assay.

Nucleotide sequences of the primers used in the RT-qPCR assay

Gene	Nucleotide sequences
PsK1-F	5′-CGCGATGTAAAATCAGCAAACATATTG-3′
PsK1-R	5′-CGTCACCATATTGAGCATATTCTGG-3′
PsSym10-F	5′-GTACTTCATTGGCGGAGACTG-3′
PsSym10-R	5′-CCATAAGTTTCACAAGATTTCCAT-3′
SlNSP2-F	5′-AAGGCCGATAGGAGACGAAGAAGG-3′
SlNSP2-R	5′-CCCCACCCCACTCAACCACTC-3′
SlMAPK6-F	5′-CGCGCTTGCTCATCCTTACCTA-3′
SlMAPK6-R	5′-GTGCTGGTATTCGGGATTAAATG-3′
SlD27-F	5′-GCTACCACAGGATTAAGAAACAAG-3′
SlD27-R	5′-CCAACTAGCCAAGGAAAGAAGAT-3′
SlRAM1-F	5′-GGAAGCGGTCAGGGAAACAGG-3′
SlRAM1-R	5′-CCAGGAACCGACCCAGGAAATAC-3′
SlGADH-F	5′-TGAGAATCAACACACTTCTCCAAGG-3′
SlGADH-R	5′-GCATTAAGAATTTCCCCAGAGGTC-3′

The RT-qPCR assay revealed the *PsSym10* gene expression only in the roots of plants transformed with the pSlEXT1::*PsSym10-3xFLAG*::T35S construct (Fig. 3). Similarly, the expression of the *PsK1* gene was detected only in the roots of plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct (see Fig. 3). Therefore, agrobacteria-mediated transformation and transfer of the *PsSym10* and

PsK1 pea genes into the DNA of tomato root cells stimulated the expression of these genes.

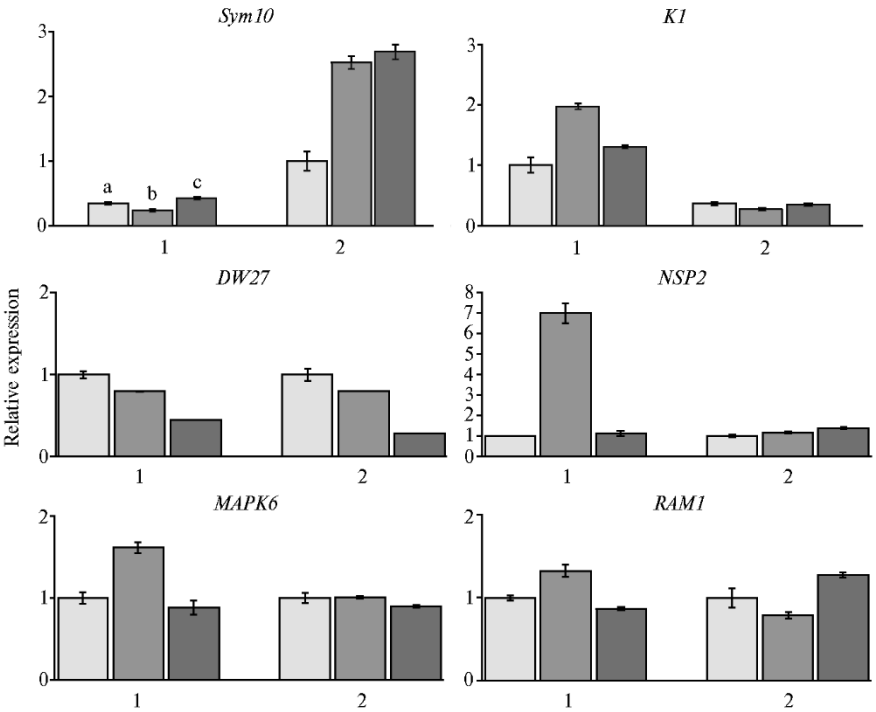

Fig. 3. Relative expression of the genes *PsSym10*, *PsK1*, *SlD27*, *SlNSP2*, *SlMAPK6*, and *SlRAM1* in the roots of composite tomato (*Solanum lycopersicum* L., cv. Carmello) transformed with the constructs pSlEXT1::*PsK1-RFP*::T35S (1) **and pSlEXT1::*PsSym10-3xFLAG*::T35S** (2) upon inoculation with ***Rhizobium leguminosarum* bv. *viciae* CIAM1026:** a — control (without inoculation), b — 7 days after inoculation, c— 21 days after inoculation. The expression levels are normalized to the tomato gene *GADH* expression, the values are shown as a relative expression level upon inoculation to the control without inoculation. Bars mean standard errors of mean (±SEM) for three analytical replicates. For each assay, RNA was extracted from 3-5 transgenic roots.

In the roots of composite tomato plants, there was a significant (approximately 2.0-2.5-fold) increase in the expression of the *PsSym10* gene both 7 days and 21 days after inoculation with rhizobia as compared to control. The increase in the expression of the *PsK1* gene turned out to be the most significant 7 days after inoculation. After 21 days, the expression of the *PsK1* gene decreased, but remained higher than in the control roots without inoculation. Therefore, the pSlEXT1 extensin gene promoter can activate both *PsSym10* and *PsK1* pea genes in composite tomato plants.

It is known that the extensin gene promoter is regulated by ethylene [23]. It also was found that the inoculation of leguminous plants with rhizobia in the roots can increase the ethylene production [24]. An increase in the ethylene content in plant roots upon rhizobia inoculation can explain the relatively high expression of pea genes under the extensin gene promoter. This promotor can be convenient for studying the effect of transferred legume genes on tomato plants upon inoculation with rhizobia.

It is known that binding of signaling molecules to receptors localized in the plasma membrane of plant root cells activates the components of the "common" signaling pathway (CSP) [25]. The roots of tomato plants are capable of perceiving Nod factors, and in this case, under the influence of signaling molecules, a change in the ion flux through the membrane and its depolarization can

be activated [18]. However, it remained unknown whether Nod factors can activate CSP components in non-leguminous plants.

It was of interest to reveal i) whether the transfer of pea genes encoding two receptors for Nod factors will affect the expression of markers normally activated by CSP components and ii) whether the CSP components will be activated in the composite tomato plants under the influence of transferred receptor genes in response to recognition of rhizobia Nod factors. We compared the expression of marker genes for plant symbiosis with AM fungi activated by signal transduction, using the transformed tomato roots without inoculation and upon inoculation with rhizobia. Previously, we carried out a search for gene sequences in tomato, which may be homologues of genes of leguminous plants activated in symbiosis with AM fungi. These are the genes of β-carotene isomerase DWARF27 (D27), transcription factor Nodulation Signaling Pathway 2 (NSP2), transcription factor Required for Arbuscular Mycorrhization (RAM1), and Mitogen-Activated Protein Kinase (MAPK6) [12]. The expression of marker genes was analyzed in transgenic roots of the composite tomato plants on days 7 and 21 upon inoculation with the *R. leguminosarum* bv. *viciae* CIAM1026 (see Fig. 3).

In the roots of tomato plants with the pSlEXT1::*PsSym10-3xFLAG*::T35S construct, there were no significant changes in the expression of the *SlD27*, *SlNSP2*, *SlRAM1*, and *SlMAPK6* genes (see Fig. 3). In plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct, the expression of *SlD27* and *SlRAM1* genes also did not change significantly in response to inoculation, while the expression of *SlNSP2* and *SlMAPK6* genes significantly increased (see Fig. 3). The activation of these markers may indicate the effect of the transferred pea gene *PsK1* on the susceptibility of tomato plants to inoculation with rhizobia. In the future, a more detailed analysis of morphological changes in plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct will be carried out. It may be of great interest to analyze plants transformed simultaneously with two genes, the *PsSym10* and *PsK1* in one genetic construct, since in peas both of these receptors (in the complex) are involved in the binding of Nod factors.

Thus, we managed to obtain composite tomato plants of the Carmello cultivar transformed with pea genes *PsSym10* and *PsK1* which encode receptors for Nod factors. Integration of the *Sym10* and *K1* genes into the tomato genome was carried out using the vector constructs pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S and confirmed by PCR analysis. Inoculation with the *Rhizobium leguminosarum* bv. *viciae* CIAM1026 enhanced the expression of both the *PsSym10* gene and the *PsK1* gene in the transgenic roots of composite plants. In the roots transformed with the pSlEXT1::*PsK1-RFP*::T35S construct, the expression of the *SlNSP2* and *SlMAPK6* genes which are normally activated under the influence of the components of the "common" signaling pathway selectively increased. Activation of the expression of two genes, *SlMAPK6* and *SlNSP2*, in response to inoculation may indicate the effect of the transferred pea receptor gene on the susceptibility of tomato plants to inoculation with rhizobia.

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ANALYSIS OF THE EFFECTS OF JOINT INOCULATION BY ARBUSCULAR MYCORRHIZAL FUNGI AND RHIZOBIA ON THE GROWTH AND DEVELOPMENT OF PEA PLANTS *Pisum sativum* L.

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Abstract

Co-inoculation of plants with arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria of the order *Rhizobiales* (rhizobia) can have a stimulating effect on plant growth and development. This influence can be considered as the synergistic effect of two microorganisms on a plant in a multicomponent system and as the result of the mutual influence of microorganisms on each other. However, the mechanisms underlying the mutual influence of microorganisms remain insufficiently understood. In the presented work, it was shown for the first time that in the case of joint inoculation of pea plants with fungi of arbuscular mycorrhiza and rhizobia, the method of introduction microorganisms may be important. The results may indicate the presence of competition of microorganisms for a niche in the plant during sequential inoculation. The purpose of our research was to study the possibility of selection of the effective combinations of AM and rhizobia strains for inoculation of such important agricultural crop as *Pisum sativum* L. as well as estimation of influence on the productivity of plants. In this work, we analyzed the effects of joint inoculation of pea plants *Pisum sativum* L. with the fungus *Rhizophagus irregularis* BEG144 and the rhizobial strain *R. leguminosarum* bv. *viciae* RCAM 1026. For this purpose, the level of induction of markers development of two types of symbiosis, the degree of root mycorrhization were assessed, as well as the biometric parameters of plants. The research was performed using pea seedlings of the cultivar Frisson grown under sterile conditions. The isolate of the fungus *Rhizophagus irregularis* BEG144 was used for inoculation. An inoculum was obtained from the mycorrhized roots of *Plecthrantus australis.* The inoculum was introduced into a moisture substrate before planting the pea seedlings. The scheme of sequential inoculation was used, according to which pea plants were first inoculated with AM fungi *R. irregularis* BEG144 and 7 days after cultivation, the rhizobial strain *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 were introduced into the system. The experimental scheme included the following options: without inoculation (control), *R. leguminosarum* (Rlv), *R. irregularis* (AM), *R. irregularis* + *R. leguminosarum* (AM + Rlv). 9 and 21 days after planting (2 and 14 days after bacterial inoculation), the material was collected to analyze the expression of genes - markers of the legume-rhizobial symbiosis development. On day 21 after planting, the plants were collected in order to determine their biometric parameters, as well as the markers of the symbiosis development. The lateral roots of the plants were cut off and frozen in liquid nitrogen. After isolation of the total RNA, cDNA was synthesized on the RNA template using reverse transcriptase using oligo(dT) primers. For the analysis, quantitative PCR combined with reverse transcription (RT-PCR) was performed (a CFX96 Real-Time instrument, «Bio-Rad Laboratories», USA). The total weight of plants and the weight of the root system significantly increased in case of monoinoculation with rhizobia, monoinoculation with AM fungi, as well as joint inoculation, compared with the control variant. However, no significant differences in these biometric parameters between the variant with monoinoculation with rhizobia or AM fungi and double inoculation were found. Probably, upon inoculation with several endosymbionts, competition arose between them at the stage of penetration into the plant, which led to a decrease in the intensity of plant infection with rhizobia. This was evidenced

by the absence of additional stimulation of the *Enod5* and *Sym37* gene expression during double inoculation, which are activated in the plant during the development of rhizobial infection. Upon double inoculation, we also did not reveal additional stimulation of the expression of marker genes of symbiosis with AM fungi — *PT4, TI, RAM1* and *DELLA3*. This correlated with the absence of significant differences in all biometric parameters between the variant with monoinoculation with rhizobia or AM fungi and double inoculation, which does not allow us to conclude about the positive effect of double inoculation on the growth and development of pea plants under the conditions of this experiment. The results of data analysis using the experimental scheme used may indicate the presence of competition of microorganisms for a niche in the plant, leading to a decrease in intra-root mycorrhizal colonization and the level of induction of markers that are activated during the development of rhizobial infection.

Keywords: plant-microbe interactions, rhizosphere, symbiosis, arbuscular mycorrhiza, inoculation, rhizobia, *Rhizophagus irregularis*, gene expression, *Pisum sativum*

Currently, world agriculture is facing the problem of transition from the intensive to a sustainable type aimed at environmentally friendly farming [1, 2]. The use of mineral fertilizers, which are costly and severely damaging to the environment, should be reduced [3]. The ability of most agricultural crops to form endosymbioses with mycorrhizal fungi of the phylum *Glomeromycota* (the arbuscular mycorrhiza, AM) plays a significant role in improving plant mineral nutrition, primarily phosphorus nutrition, and productivity. An alternative to the use of mineral nitrogen fertilizers can be the biological nitrogen. Symbiosis between legumes and bacteria of the order *Rhizobiales* called rhizobia plays a significant role in biological nitrogen fixation.

Co-inoculation with AM fungi and rhizobia also positively affects plants, mainly due to the improvement of mineral nutrition and inhibition of fungal pathogens [4-7]. Since the lack of phosphorus and nitrogen in the soil is one of the limiting factors, co-inoculation stimulates plant growth. In this case, the stimulating effect can be due both to the synergistic effect of microorganisms on a plant in a multicomponent system and to the influence of microorganisms on each other. However, the mechanisms underlying the mutual influence of microorganisms remain poorly understood.

It is known that AM fungi provide plants with available phosphorus which plays an important role in energy metabolism and especially in nitrogen fixation as an energy-consuming process [8]. Mobilization of phosphorus can stimulate the activity of nitrogenase and nitrogen fixation by rhizobia [9-11]. Indeed, upon co-inoculation of *Phaseolus vulgaris* L. and *Medicago arborea* L. with AM fungi and rhizobia, a significant increase in the number and weight of nodules occurred as compared to monoinoculation [12, 13]. Co-inoculation led to a greater accumulation of phosphorus and nitrogen in the bean shoots and to a better phosphorus utilization compared to control plants [12]. The positive effect of double inoculation with AM fungi and rhizobia on growth, nutrient uptake, and nitrogen fixation occurred in soybeans [4], cowpea [14], and peas [15]. The double inoculation of pea plants leads to an increase in their biomass, the number and weight of nodules, the efficiency of nitrogen fixation and transport of bound forms of nitrogen [16, 17].

The mutual influence may be due to signaling molecules that AM and rhizobia fungi exchange with the plant, i.e., Myc and Nod factors. Co-inoculation of soybean *Glycine max* (L.) Merr. with *Bradyrbizobium japonicum* 61-A-1O1 significantly enhanced the colonization of plant roots by the fungus *Glomus mosseae* [18]. The Nod factors of rhizobia can influence stimulation of the mycorrhiza formation, since plant inoculation with strains defective in the synthesis of these signaling molecules did not lead to an increase in root colonization by the fungus. The positive effect can be associated with the structural similarity of the Nod and Myc factors, since the structure of Myc factors is similar to that of Nod factor with minimal substitutions on the molecular backbone [19]. In addition, Nod

factors activate the synthesis of flavonoids in plant roots which also have a stimulating effect on root colonization by AM fungi [18].

Co-inoculation also increases in resistance to pathogens. In plants inoculated with AM fungi the incidence and severity of symptoms caused by *Rhizoctonia*, *Fusarium*, or *Verticillium*, as well as oomycetes *Phytophthora*, *Pythium*, and *Aphanomyces*, decrease [20]. The biocontrol properties of rhizobia can be associated with the release of lytic enzymes and antimicrobial secondary metabolites that suppress the pathogens [21]. With co-inoculation, the effect of AM and rhizobia fungi can be enhanced. Thus, in soybeans, double inoculation significantly reduced the signs of red crown rot. This effect correlated with the high expression of the *PR2*, *PR3*, *PR4*, and *PR10* genes which control defense reactions [22]. However, it remains unclear how AM and rhizobia fungi in a multicomponent system avoid mutual negative influence caused by the release of lytic enzymes and antimicrobial compounds.

Probably, due to the peculiarities of the mutual influence, the effect of AM fungi and rhizobia on a plant during co-inoculation varies greatly depending on the strains used. In this case, both a significant stimulation of plant growth and the absence of a pronounced effect can occur [15, 23]. For example, the yield and nitrogen content in pea plants were maximum when co-inoculated with the fungus *Glomus clarum* NT4 and the highly efficient strain *Rhizobium* LX43 [15]. On the contrary, the fungus strain *Glomus mossae* NT6 increased the yield of peas when co-inoculated only with the ineffective rhizobia strain 175P4 [15]. Consequently, through the selection of effective strain combinations of fungi and rhizobia, it is possible to influence crop yields but it is necessary to understand what mechanisms underlie this interaction.

The presented work shows for the first time that the mode of introducing fungi of arbuscular mycorrhiza and rhizobia when co-inoculating pea plants is important. Namely, with sequential inoculation, the microorganisms compete for a niche in the plant.

Our goal was to investigate the possibility and effect of co-inoculation of pea *Pisum sativum* L. seed with a combination of the arbuscular mycorrhiza fungus *Rhizophagus irregularis* BEG144 and rhizobia *Rhizobium leguminosarum* bv. *viciae* RCAM 1026, to assess the induction of markers of two types of symbiosis, the degree of root mycorrhization, and biometric parameters of plants.

Material and methods. Pea (*Pisum sativum* L., cv. Frisson) seeds were sterilized for 10 min with concentrated H_2SO_4, washed 3 times with sterile distilled water, and germinated in Petri dishes with 1% agar (4 days at room temperature in the dark).

For inoculum of *Rhizophagus irregularis* BEG144 (provided by the International Bank for the Glomeromycota, Gidon, France), the mycorrhized roots of *Plectranthus australis* were washed in running tap water, rinsed 3 times with distilled water, cut into fragments ~ 1 cm long, and examined under a microscope to detect fungal propagules (vesicles and spores). The inoculum (1.3 g per pot) was introduced into a moistened substrate to a depth of 3 cm before planting pea seedlings. In 7 days, *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 was introduced. For the inoculum, the bacteria were cultured at 28 °C on solid TY medium [24] with streptomycin (500 μg/ml) and washed off the plates with autoclaved tap water. The resultant suspension was diluted to $OD_{600} = 0.5$ and used for inoculation (2 ml per pant).

The plants were grown under the controlled conditions using clay marl added with 1 g/l $CaCO_3$ as a substrate (a MLR-352H phytotron, Panasonic, Japan; 16 h day/8 h night, 21 °C, and 60% relative humidity). Pots with the substrate

were pre-sterilized by autoclaving (60 min, 134 °C, 0.22 MPa). A designed nutrient solution (without phosphorus and with a reduced nitrogen concentration) was used for plant feeding. The solution contained macroelements (mmol per l of the substrate) NH_4NO_3 0.16, or 1/10 of the norm, $MgSO_4 \cdot 7H_2O$ 0.51, and K_2SO_4 0.72, and microelements (μmol per l of the substrate) H_3BO_3 9.19, $MnSO_4 \cdot 5H_2O$ 2.28, $ZnSO_4 \cdot 7H_2O$ 0.19, $CuSO_4 \cdot 5H_2O$ 0.08, $(NH_4)_2MoO_4$ 0.03, $CoCl_2 \cdot 6H_2O$ 0.03, and NaFe-EDTA 8.36. Top dressing was carried out once during planting pea seedlings; watering was carried out with distilled water as needed.

The experimental design included no treatment (control without inoculation), inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 (Rlv), inoculation with *Rhizophagus irregularis* BEG144 (AM), and co-inoculation with *R. irregularis* + *R. leguminosarum* (AM + Rlv).

Nine and twenty-one days after planting (2 and 14 days after bacterial inoculation), plant material was collected to analyze the expression of markers of legume-rhizobial symbiosis (genes *Sym10*, *NIN*, *Enod5*, and *Sym37*). On day 21 after planting (day 14 after inoculation with rhizobia), the plants were collected to measure their biometric parameters, to analyze the expression of markers of AM fungus inoculation (genes *PT4*, *TI*, *RAM1*, and *DELLA3*), and to assess the development of symbiosis. The development of AM was assessed using light microscopy as described previously [25].

The lateral roots were cut off and frozen in liquid nitrogen for isolation of total RNA using NucleoSpin® RNA columns as per the manufacturer's method (Macherey-Nagel, Germany). cDNA on the RNA template was synthesized using RevertAidH minus reverse transcriptase (Thermo Scientific, USA) and oligo(dT) primers (Evrogen, Russia). For the analysis, quantitative PCR combined with reverse transcription (RT-qPCR) (CFX96 Real-Time PCR Detection System, Bio-Rad Laboratories, USA) was run as follows: 30 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (40 cycles); the primers are

PsSym10-F — 5′-GTACTTCATTGGCGGAGACTG-3′;
PsSym10-R— 5′-CCATAAGTTTCACAAGATTTCCAT-3′;
PsNIN-F — 5′-CCGCAAAGAGCATCGGTGTATG-3′;
PsNIN-R — 5′-GCATAGAAAGATCCAATCTGTATAGC-3′;
PsPT4-F — 5′-CTTCACGTGCCATGTTCATC-3′;
PsPT4-R — 5′-GCGTCGGAAACAGCTCC-3′;
PsTI-F — 5′-ACCTTACAGCGTGAGCCTATAAGA-3′;
PsTI-R — 5′-GCGGCCGAGGTACGAAAGGTG-3′;
PsRAM1-F — 5′-GTCCATGATAAGAGACCAAGCACC-3′;
PsRAM1-R — 5′-GGAGGAAGATAATGGAAGGGAAAG-3′;
PsDELLA3-F — 5′-GCAATATAGAATTAACCGCCACAAC-3′;
PsDELLA3-R — 5′-CGGATGAGCGGGACAACC-3′.

The amount of mRNA was normalized to two constitutively expressed genes encoding ubiquitin and actin. Three independent biological replicates were analyzed.

The results were statistically processed (SigmaPlot 12.0 software, SPSS Inc., USA). The means (*M*) and standard errors of the means (±SEM) were calculated. One-Way ANOVA with multiple pairwise comparisons using the post hoc Tukey test (ANOVA post hoc test) were used to assess the significance of differences between the treatments. Differences were considered statistically significant at $p < 0.05$.

Results. We used *Sym10* and *NIN* genes activated in plant roots on days 1-2 in response to inoculation [26] as markers of early stage of establishing symbiosis in pea plants upon inoculation with rhizobia. The *Sym10* gene encodes a LysM-containing receptor-like kinase involved in the recognition of the signaling

molecules of rhizobia — lipochito-oligosaccharides (Nod factors). The activation of the signaling cascade under the influence of the *SYM10* receptor leads to the development of nitrogen-fixing nodules on the plant roots. In the signal transduction, the expression of the *NIN* (Nodule Inception) gene which encodes a transcription factor and thus participates in the initiation of the development of symbiosis of peas with rhizobia, significantly increases.

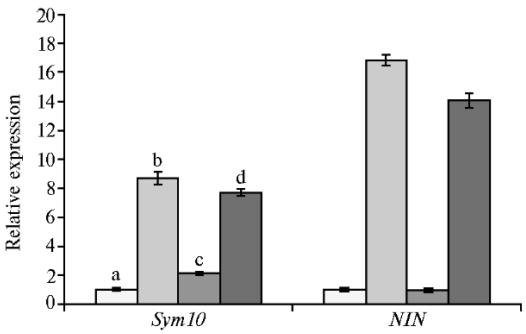

Fig. 1. Expression of the genes *Sym10* (receptor of rhizobia signaling molecules) and *NIN* (the main transcription factor of the signaling pathway) in pea (*Pisum sativum* L., cv. Frisson) roots 9 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 2 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminosarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

Monoinoculation with rhizobia caused a significant increase in the expression of the *Sym10* and *NIN* genes as compared to the non-inoculated control on day 9 (Fig. 1). In plants inoculated with AM fungi *R. irregularis* BEG144, no significant changes occurred in the *Sym10* and *NIN* expression which indicates the specific activation of these genes only in response to the recognition of signaling molecules of rhizobia (see Fig. 1). The expression of *Sym10* and *NIN* genes remained high upon co-inoculation with Rlv and AM fungi (see Fig. 1). In our opinion, this indicates that at the initial stages of formation and development of the multicomponent system, the plant effectively distinguishes the signals of rhizobia. They stimulate early responses in plants, including changes in ion fluxes across the plasma membrane and its depolarization, the production of reactive oxygen species, and the activation of channels that regulate the calcium flow into plant cells (Ca^{2+} influx).

The expression of genes *PT4*, *TI*, *RAM1*, and *DELLA3* that we used as markers for symbiosis with AM fungi increases most significantly on days 21-28 after inoculation with AM fungi [27]. The *PT4* gene encodes a phosphate transporter [17] the activation of which is associated with the later steps of the development of symbiosis with AM fungi when formation of arbuscules and vesicles occur. Transcriptomic profiling of the roots of pea plants inoculated with the AM fungus revealed a transcriptional inhibitor encoded by the *TI* gene [17]. Of transcription factors, the expression of RAM1 regulator was found to increase during the symbiosis with AM fungi [27]. DELLA proteins are regulators of plant response to gibberellins; they are directly involved in the control of the development of both nitrogen-fixing symbiosis and symbiosis with AM fungi [28, 29]. Proteins interact with the main transcription factors of signaling pathways — NSP2, IPD3, and RAM1, which stimulates the activation of target genes [29]. Earlier, we showed that in pea plants, symbiosis with AM fungi increases the expression of one of the genes of this family, DELLA3 [27].

On day 21 after planting, we revealed a significant increase in the expression of the *PT4* and *TI* genes upon monoinoculation by *R. irregularis* BEG144 and co-inoculation (AM + Rlv) (Fig. 2). Similarly, the expression of the *RAM1* and *DELLA3* genes increased with AM and AM + Rlv treatments. This indicated the ability of plants to respond to inoculation with AM fungi when applied together with rhizobia; however, we did not detect any additional stimulation of marker

gene expression during co-inoculation.

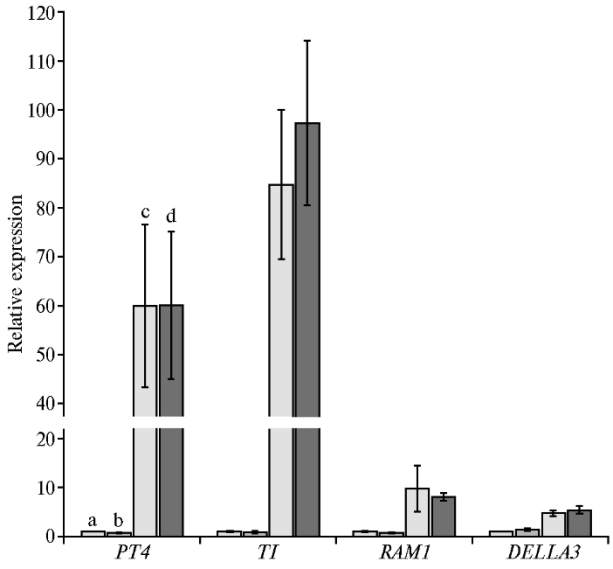

Fig. 2. Expression of genes *PT4* (a phosphate transporter), *TI* (a transcriptional inhibitor), *RAM1* (a transcription factor), and *DELLA3* (a regulator of plant response to gibberellins) in pea (*Pisum sativum* L., cv. Frisson) roots 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminoussarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

On day 21, we assessed expression of the genes *Enod5* and *Sym37* that can be activated during legume-rhizobial symbiosis in peas. *Enod5* encodes nodulin which is activated in a specific way in pea root cells after penetration of infection threads [30, 31]. The *Sym37* gene encodes a receptor that is necessary for the development and spread of infection threads in pea roots [32].

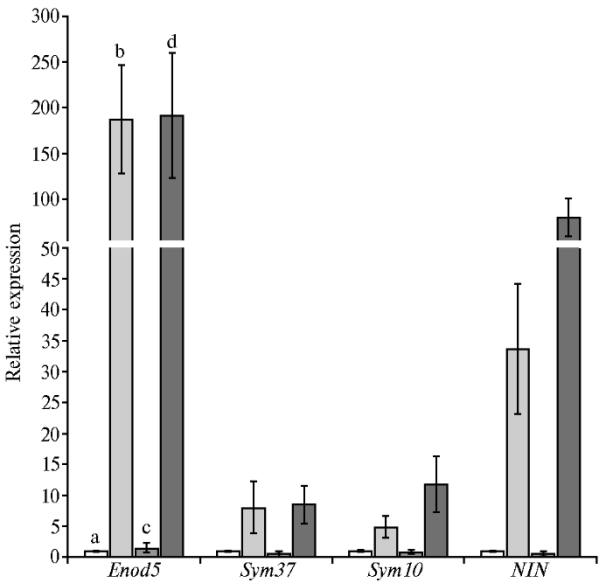

Fig. 3. Expression of genes *Enod5* and *Sym37* that can be activated during development of legume-rhizobial symbiosis in pea (*Pisum sativum* L., cv. Frisson) roots 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: a — without inoculation (control), b — *R. leguminosarum*, c — *R. irregularis*, d — *R. irregularis* + *R. leguminoussarum*. Bars show standard errors of the mean (±SEM) for three analytical replications. The experiment was arranged in three biological replicates (the data of one replicate are shown).

On day 21 after planting, the expression of the *Enod5* and *Sym37* genes, as well as *Sym10* and *NIN* genes increased upon monoinoculation with rhizobia (Fig. 3). No changes occurred in the expression of these genes with *R. irregularis* BEG144 inoculation (see Fig. 3). With AM + Rlv, the expression of the *NIN* gene increased compared to the Rlv monoinoculation. However, upon the co-infection, the expression of the genes *Enod5* and *Sym37* associated with the control of the infection development remained similar to that upon monoinoculation with rhizobia. Expression of the *Sym10* gene also did not change significantly upon co-inoculation of pea plants with the fungus and rhizobia.

The development of infection in legume-rhizobial symbiosis and symbiosis with AM fungi is under the host plant control. Probably, upon successive inoculation with several endosymbionts, competition arises between them at penetration into the plant, which leads to a decrease in the intensity of infection with rhizobia. In our opinion, the evidence to support this assumption is that co-inoculation did not additionally stimulate the expression of the genes *Enod5* and *Sym37* activated during the rhizobial infection.

With Rlv monoinoculation, there was a pronounced tendency to an increase in the total biomass of plants compared to the control ($p = 0.072$) (Fig. 4).

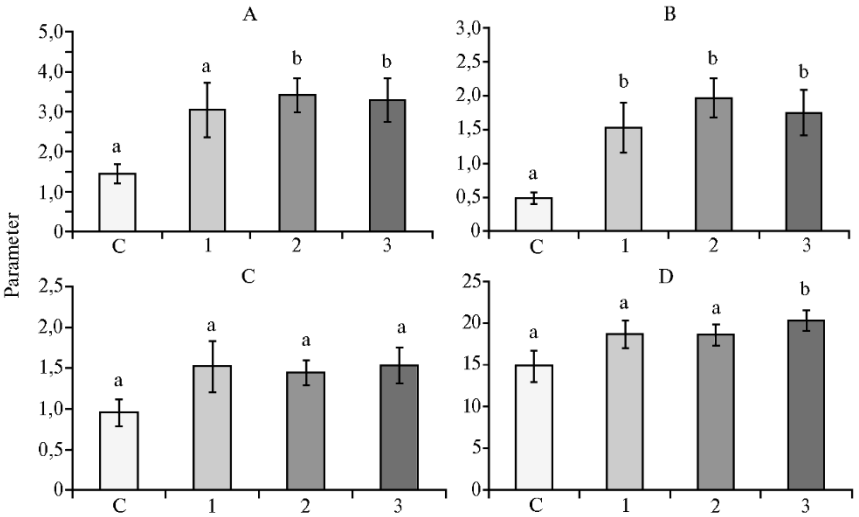

Fig. 4. Total biomass, g (A)**, root weight, g** (B)**, aboveground biomass, g** (C)**, and root length, cm** (D) **in pea (*Pisum sativum* L., cv. Frisson) plants 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026:** C — without inoculation (control), 1 — *R. leguminosarum*, 2 — *R. irregularis*, 3 — *R. irregularis* + *R. leguminosarum*. The arithmetic mean values for n = 12-15 are presented. Bars show standard errors of the means (±SEM). Different letters indicate statistically significant differences ($p < 0.05$). To assess the significance of differences between the treatments, one-way ANOVA post hoc test was used.

A significant change ($p < 0.05$) of the total plant biomass occurred both upon monoinoculation with AM fungus and co-inoculation ($p < 0.05$) (see Fig. 4, A). The root weight significantly ($p < 0.05$) increased upon inoculation with Rlv, AM, and AM + Rlv as compared to the control. However, Rlv, AM, and AM + Rlv did not differ significantly in root weight (see Fig. 4, B). Weighing of the aerial parts did not reveal significant differences between the control and mono- (Rlv or AM) or co-inoculation (AM + Rlv) (see Fig. 4, C).

Upon inoculation with Rlv and AM, there was a tendency to an increase in the plant root length compared to the control ($p = 0.08$ and $p = 0.09$, respectively); however, only co-inoculation led to a significant increase ($p < 0.05$) (see Fig. 4, D). Any inoculation treatment had no significant effect on the length of the aerial parts.

The obtained results allow us to conclude that in our experiment, monoinoculation with rhizobia or AM fungi was effective. Rlv and AM monoinoculation increased the biomass of the plant roots but not the aerial parts. Upon co-inoculation, a slight increase in the root length ocurred. However, the absence of significant differences in plant weight and root weight between the Rlv or AM monoinoculation and co-inoculation does not allow us to draw a conclusion about the positive effect of double inoculation on the growth and development of pea plants.

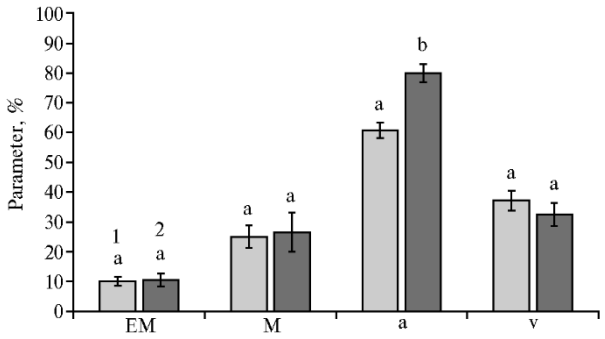

Fig. 5. Developemt of arbuscular mycorrhiza in pea (*Pisum sativum* L., cv. Frisson) plants 21 days after planting into substrate with *Rhizophagus irregularis* BEG144 and 14 days after inoculation with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026: EM — intensity of external mycorrhization, M — intensity of in-root mycorrhization, a — abundancy of arbuscules in mycorrhized root fragments, v — abundancy of vesicles in mycorrhized root fragments; 1 — *R. irregularis*, 2 — *R. irregularis* + *R. leguminosarum*. The arithmetic mean values for n = 12-15 are presented. Bars show standard errors of the means (±SEM). Different letters indicate statistically significant differences ($p < 0.05$). To assess the significance of differences between the treatments, one-way ANOVA post hoc test was used

On day 21, all plants inoculated with rhizobia formed pink nodules (on average, 180±35 per plant with Rlv monoinoculation and 228±55 per plant with AM + Rlv). There were no statistically significant differences in the number of nodules between these two treatments.

In both fungal inoculation (AM and AM + Rlv), the plants had approximately the same intensity of external root colonization (EM%) with *R. irregularis* hyphae (approximately 10%) (Fig. 5). This testified to the good quality of the mycorrhizal inoculum. The intra-root mycelium developed quite intensively in both variants, however, for AM and AM + Rlv, no significant differences in the intensity of intra-root colonization (M%) occurred (see Fig. 5). In the mycorrhized root segments, arbuscules intensively developed. However, the relative number of arbusules (a%) was significantly higher ($p < 0.05$) for co-inoculation than for AM monoinoculation, which indicates a more intensive exchange of nutrients between fungal and plant cells (see Fig. 5). Inoculation with rhizobia did not affect the relative number of vesicles (v%) serving as storage organs of AM fungi (see Fig. 5). Without fungal inoculation (in control and in Rlv inoculation), no structures of AM fungi were found, which indicates the absence of cross-contamination with these microorganisms.

Overall, based on the analysis performed, it can be concluded that legume-rhizobial symbiosis and symbiosis with AM fungi developed in the system under study. In this regard, the data obtained on the expression of genes markers of symbiosis are adequate. However, the analysis of the double symbiosis showed that when using the AM + Rlv sequential inoculation, there is no significant increase in intra-root mycorrhizal colonization, which is probably due to the competition between microorganisms for a niche in the plant or to stimulation of a systemic response from plants.

Numerous publications show that AM fungi and rhizobia act as synergists, stimulating plant growth via improved mineral nutrition and inhibition of fungal pathogens [4-7]. However, several works revealed that competition for a niche in a plant can be the reason for a decrease in the efficiency of root colonization by AM fungi. AM *Glomus* sp. R-10 commercial inoculum used to increase yields of soybean *Glycine max* (L.) Merrill had a negative effect on the root colonization by endogenous strains of fungi well adapted to the conditions of plant growing [33]. Similarly, in carrot roots which were cultivated in vitro, competition for a niche was the reason for a decrease in intra-root colonization upon co-inoculation with AM *Rhizophagus irregularis* and *Glomus aggregatum* as compared to monoinoculation [34]. Therefore, increased competition between symbionts can

affect the intensity of intra-root colonization by AM fungi. This determines the interest in studying the mutual influence of symbionts on plants.

The mutual influence of AM fungi and rhizobia in a multicomponent system may be quite important, especially with the sequential inoculation we used. Our data indicate that not only combinations of certain strains of fungi and rhizobia are important to achieve a positive effect on plants but also the scheme of co-inoculation. In the future, it is necessary to identify the conditions, decreasing competition, and to assess whether such changes in mycorrhizal colonization can be a marker of the effectiveness of plant—AM fungi interaction during co-inoculation.

Thus, the level of the induced expression of markers for two types of symbiosis, the root mycorrhization intensity, and the plant biometric parameters draw us to the conclusion that a two-step inoculation of pea plants (cv. Frisson), first with *Rhizophagus irregularis* BEG144 and then with *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 failed to reach the stimulating effect characteristic of co-inoculation if compared to monoinoculation. Our findings may indicate the competition of microorganisms for a niche in the plant or the activation of systemic mechanisms that prevent the enhancement of intra-root colonization by symbionts.

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A NANOSILICON PREPARATION IS SUPERIOR TO A BIOLOGICAL PREPARATION AND A CHEMICAL PREPARATION IN ACTIVITY TOWARDS PHOTOSYNTHETIC PRODUCTIVITY AND YIELD PARAMETERS OF SPRING WHEAT

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Abstract

Wheat is widely used as a food, technical and feed crop. Increased wheat yields can be achieved by mitigating biotic and abiotic stresses using a variety of technologies that include trace elements and growth regulators. Nanosilicon microfertilizer (NanoSilicon LLC, Russia) is an environmentally friendly product containing 50 % of pure colloidal-sized crystalline silicon. This work, for the first time, confirms the positive effect of the Nanosilicon preparation on photosynthetic potential and the net productivity of photosynthesis, the synthesis of chlorophyll, carotenoids and sugars and shows an advantage of Nanosilicon over the pesticide Vincite and an experimental biological product. Under the influence of Nanosilicon, the component structure of the spring wheat yield changed, namely, the number of productive stems, ears and 1000-grain weight increased. Our goal was to examine the effect of Nanosilicon preparation on spring wheat photosynthetic productivity and yield components in the conditions of the Orel region and to compare the effect of Nanosilicon with that of a chemical pesticide and a bioactive preparation. The experiment design included four treatments of spring wheat (*Triticum aestivum* L.) cv. Darya seeds (Federal Research Center for Grain-Legumes and Cereals, Streletskoe village, Oryol region, 2016-2019). The seeds were soaked for 2 hours before sowing in water, in chemical pesticide Vinzit (two controls), in a novel biological product based on buckwheat bioflavonoids, and in Nanosilicon concentrate (tests). During vegetation, the control and test treatments were twice applied to the growing plants at tillering and at stem extension phases. The energy of seed germination and germination rate were determined, the development of seed infections was assessed. The phenological phases (three leaves, tillering, stem extension, earing, flowering, milk ripeness, and full ripeness of the grain) were recorded. Photosynthetic potential (PP), photosynthetic productivity and net photosynthetic productivity (NPP) were evaluated, leaf area and the pigment content were measured. It was found that pre-sowing treatment of spring wheat seeds with Nanosilicon contributed to an 18.5 % increase in germination energy and a 5.5 % increase in germination rate as compared to the control treatments. Due to the Nanosilicon application, the plants were taller, resulting in more leaves until the end of the growing season, which indicates a longer leaf life compared to controls. The leaf area was 20.0 % larger at the earing-flowering period compared to the control (water), that was, 14.6 % larger for the biopreparation and 8.3 % larger for the pesticide Vincit. Photosynthetic capacity for control (water), Vincit, Nanosilicon, and the biopreparation was 633360, 686022, 1560384, and 1104894 $m^2 \cdot$ day/ha, respectively. NPP value for Nanosilicon was greater as compared to the controls, by 60-80 % for water and by 22.2 % for Vincit. The amounts of chlorophylls and carotenoids in plants were the greatest at the earing—flowering phase. Under the influence of Nanosilicon and the biological preparation, the synthesis of pigments increased by 20-30 % compared to the controls. Nanosilicon contributed to an increase in the synthesis of sugars in the process of photosynthesis to a lesser extent than the biological product, which can be explained by the difference in the distribution of assimilates and a large accumulation of proteins. The advantages of the Nanosilicon over the bioactive preparation in the number of grains and the 1000-seed weight were minor. Under the effect of Nanosilicon, the number of productive stems increased by 33.7 %, the number of ears by 38.7 %, the ear weight by 26.8 %, the number of grains per ear by 19.2 grains, and the 1000-grain weight by 19.7 % as compared to the control. These indicators for the bioactive preparation were slightly lower than for Nanosilicon,

but higher than in control treatments. For four years, the grain yield under the influence of Vincite was approximately 8 % higher compared to the control (water) and from 9 to 16.6 % higher due to Nanosilicon and the bioactive preparation.

Keywords: spring wheat, Nanosilicon, biological product, germination energy, germination rate, net photosynthetic productivity, yield components

Wheat, a food, industrial and fodder crop is grown on 30.3% of the grain sown area [1-4] in a wide range of climatic conditions and soils [5-7]. However, Trnka et al. [8] reported about various adverse weather events that can significantly affect wheat productivity in Europe. Climate change will lead to a reduction in the production of major cereals, with the exception of millet [9]. Technologies that mitigate the effects of biotic and abiotic stresses should increase wheat yields.

In recent decades, researchers and commercial farmers have paid significant attention to plant biostimulants and trace elements, in particular silicon. Silicon weakens the negative effects of abiotic stresses (metal toxicity, salinity, water stress, and temperature) and can reduce biotic stress [10-12]. The use of silicon fertilizers significantly increases plant resistance to diseases and pests. Silicon activates protective mechanisms, e.g., the synthesis of phytoalexins, antioxidant defense enzymes, and jasmonic acid signaling [13-15]. Silicon fertilizers have positive effects on various soils when growing rice, sugarcane, barley, sorghum, maize, wheat, oats, rye, sunflowers, beans, broad beans, soybeans, clover, alfalfa, millet, tomatoes, cucumbers, tobacco, sugar beets, lemons, tangerines, grapes, apples, and melons [16-18]. Under the influence of silicon, the intensity and productivity of photosynthesis increases. Meanwhile, the contribution of the traits that determine the production process in plants, which is carried out due to the functioning of the photosynthesizing system, is still not clear [19].

Additional photosynthesis can occur due to an increase in its daily duration or in leaf area. In grain crop, an increase in the potential number of grains and their size results from an increase in photosynthesis [6, 20, 21].

Nanosilicon microfertilizer (NanoKremniy LLC, Russia), a promising plant stimulant for grain crops, is an environmentally friendly product containing 50% pure colloidal crystalline silicon. Polyethylene glycol (food additive E1521), 6% iron, 1% copper, 0.5% zinc, 20% humic acids, 8% fulvic acids, 0.02% calcium, 0.01% boron serve as stabilizers. Unlike ordinary silicon, Nanosilicon which consists of active silicon particles of 5 nm or larger without impurities, can be completely assimilated by plants [22]. Studies conducted on spring wheat [22-24], winter wheat [25] and winter barley [26] showed that Nanosilicon contributed to an increase in the grain number per ear and a 34-35% increase in yield upon seed treatment and double spraying plants.

Recent years have witnessed growing interest in regulation and modification of physiological processes in plants, including optimized use of sunlight throughout the growing cycle. Though the dry matter production is a result of photosynthesis, it is very difficult to find a relationship between this process and the yield size. An increase in the efficiency of photosynthesis and crop yields is due primarily to the genetic improvement but also to proper variety-specific growing and farming technology and plant stimulants.

This work is the first to show the positive effect of the Nanosilicon on the photosynthetic potential, net productivity of photosynthesis, the synthesis of chlorophyll, carotenoids, sugars and the advantage of this preparation over the pesticide Vincit, KS, and a biological stimulant. Under the influence of Nanosilicon, the structure of the spring wheat crop changed, namely, the number of productive stems, ears and the 1000-grain weight increased.

Our goal was to study the effect of the Nanosilicon on the photosynthetic productivity and structure of the spring wheat yield in the Orel region and to

compare the action of Nanosilicon with a chemical pesticide and an experimental bioactive preparation.

Materials and methods. Field experiments were carried out on spring wheat (*Triticum aestivum* L.) variety Daria of the (Federal Research Center of Legumes and Groats, Streletskoye village, Orel Province, 2016-2019). The variety is included in the State Register of Breeding Achievements approved for use in the Central and Central Black Earth Regions (2016). The climate of the territory is moderately continental with a sufficient amount of heat and moisture, but precipitations are unevenly distributed. The soils of the experimental site are dark gray forest, medium loamy. In controls and treatments, 7 m^2 plots were used in four replicates. Seeds were soaked for 2 hours before sowing. During the growing season, vegetative plants were twice sprayed (at tillering and stem extension phases).

In two controls, the seeds were soaked in water (10 l/t) or in the Vincit, KS (Cheminova A/S, France, 1.5 l/t) with the use of 10 l/t working solution. For an experimental biological (Orel State Agrarian University, Russia), the dosages were 1.56 ml/t (working solution 10 l/t) for seed soaking and 3.12 ml/ha (working solution 200 l/ha) for plant spraying. For Nanosilicon (LLC NanoKremniy, Russia), the dosages were 150 g/t (working solution 10 l/t) for seed soaking and 50 g/ha (working solution 200 l/ha) for plant spraying [27].

Germination energy (percentage of seeds germinated on day 3) and seed germination rate (percentage of seeds germinated on day 7) were determined according to GOST 12038-84 "Seeds of agricultural crops. Methods for determining germination" (Mocow, 2004). Seed infections was assessed according to GOST 12044-93 "Seeds of agricultural crops. Methods for determining the incidence of diseases" (Mocow, 2011). Seed contamination was determined on day 10 in germinators. The total infection percentage was expressed compered to 100% infection.

Phenological observations covered the development stages of 2-3 leaves, tillering, stem extension, heading, flowering, milk ripeness, and full grain ripeness. The net productivity of photosynthesis was determined according to Nichiporovich [28] and calculated based on the increase in dry biomass over the test period per the average leaf area [29]. The leaf area was measured according to Moiseev et al. [29]. For examination, 10 plants were taken for each control and treatment.

The content of pigments in plants was determined according to V.F. Gavrilenko et al. [30] after homogenizing the leaves in a porcelain mortar and extracting with 100% acetone. The concentration of pigments in the extracts was measured by absorption at $\lambda = 662$ nm (chlorophyll a), $\lambda = 644$ nm (chlorophyll b) and $\lambda = 440.5$ nm (carotenoids) (an automated single-beam spectrophotometer SF-56, Lomo- Spectrum, Russia).

The tables and figures show the arithmetic means (*M*) and standard errors of the means (±SEM). The significance of the differences was determined by the Student's *t*-test at $P = 0.95$. For data processing, we used the methodological recommendations of B.A. Dospekhova [31]. The paper presents data on photosynthesis obtained in 2019, and data on yield under the influence of Nanosilicon and an experimental biological product for all four years of the study.

Results. In 2019, the phases of development of spring wheat fell on the following dates: stage of the 2nd leaf (shoots) on May 6, tillering on May 20, tillering—the beginning of stemming on May 31, heading—the beginning of flowering on June 13, end of flowering on June 23, and beginning of ripening on July 5.

The minimum seed infection (0.8%) occurred with the use of the agrochemical disinfectant Vincite, KS. In the control without treatment, the infection varied over years within 8.3-12.6%, when treated with Nanosilicon within 7.8-9.2%, and with an experimental biological within 6.4-9.5%.

In lab tests with pre-sowing seed treatment, the maximum increase in

germination energy by 18.5% and germination rate by 5.5% ($p < 0.05$) compared to control occurred upon application of Nanosilicon and the experimental biological. In the field trials, this difference was about 10%. Apparently, the increase in seed germination rate under the influence of silicon was due to the stimulation of root growth. Growth regulators, penetrating plant cells, change the activity of physiological processes, primarily the synthesis of hormones and enzymes, promoting cell division, root growth, and activation of aquaporin genes [32-34].

Photosynthetic productivity depends on physiological and biochemical processes that determine the size and quality of the yield, but there is still no clear understanding of how these processes are regulated and may be controlled [35, 36]. Heyneke et al. [37] emphasize the important role of metabolic pathways not directly involved in photosynthesis. The pathways that attract main attention are those ensuring a better carbon assimilation, an increase in the efficiency of photosynthesis and, as a result, the higher yield of biomass [38]. For example, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo), a fundamental carbon fixation enzyme, is extremely ineffective, and many strategies to improve the photosynthetic capacity of plants are focused on overcoming the limitations of the enzyme by improving its activity and regulation [39].

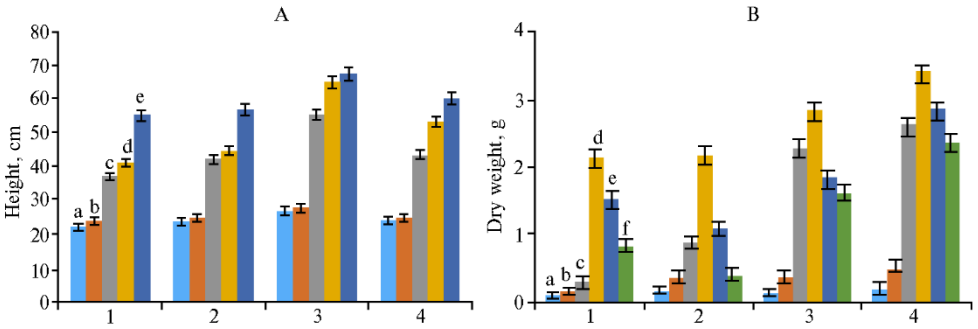

Fig. 1. Heigh (A) **and dry weigh** (B) **of spring wheat (*Triticum aestivum* L., cv. Daria) plants at 2nd leaf (seedings)** (a)**, tillering** (b)**, tillering—beginning of stem extension** (c)**, tillering—beginning of heading** (d)**, end of heading** (e)**, and beginning of ripening** (f) **stages as influenced by various treatments:** 1 — water, 2 — Vincite, KS desingectant, 3 — Nanosilicon, 4 — an experimental biological (n = 10, M±SEM; Streletskoe settlement, Orel Province, 2019).

Starting with wheat seedlings, there was an advantage in plant development due to Nanosilicon application as compared to the controls (Fig. 1). Plants from seeds treated with an experimental biological lagged somewhat behind, but were significantly more powerful than the control plants and exceeded them in dry weight. The use of Nanosilicon and biological product provided the greatest and significant excess compared to control (by 27-34%, $p < 0.05$).

The total leaf area during the beginning of the tillering—beginning of heading stages was 15834.00 m^2 for the control (water), reaching 17150.55, 19009.60, and 18141.10 m^2 for Vincite, KS, Nanosilicon, and the experimental biological, respectively. Spring wheat reached the largest leaf areas with Nanosilicon and the biological, exceeding the control (water) by 20.0 and 14.6%.

According to Mokronosov [40] and Nikitin [41], in plants that have more leaves, proper agrotechnologies and a shorter growing season can provide greater productivity even with a low intensity of photosynthesis. However, in our opinion, with an increase in the assimilating leaf area, it is worth to consider the total amount of chlorophylls, and hence the nutritional value of the crop. Meanwhile, the increase in leaf area per hectare for the entire growing season (the photosynthetic potential PP), is an indicator of the duration of the photosynthetic activity.

The sowings with PP of 2 million $m^2 \cdot$ day/ha are recognized optimal [42, 43].

With Nanosilicon and the experimental biological, the PP of spring wheat crops increased during the growing season due to an increase in the leaf area. In control (water) and upon treatments with Vincite, KS, Nanosilicon, and the biological, the PP values were 633360, 686022, 1560384, and 1104894 $m^2 \cdot$ day/ha, respectively.

The leaf area correlates with plant biomass which was the largest with Nanosilicon and the biological (see Fig. 1).

The use of growth regulators and biologicals affects the crop production process, induces plant immunity and allows people to have environmentally friendly food [44]. Pre-sowing treatment with growth regulators has a positive effect on the yield and baking properties of the products, including the protein and starch content, the amount and quality of gluten, and the bulk density of grain [45]. In corn subjected to salt stress and cold shock, application of salicylic acid, mannitol, and thiourea regulates plant growth and responses to oxidative stress, increases growth rate, leaf surface index, plant height, grain yield, and total dry matter accumulation [46, 47].

The final yield depends on other factors, among which the variety, soil and climatic conditions play a fundamental role. The formation of productivity is determined by the net productivity of photosynthesis (NPP) which depends on the assimilation surface of plants [48]. To increase the yield potential, it is necessary to increase the total biomass, but if sunlight during the growing season is already fully used, then an increase in biomass requires photosynthesis occurred in a more efficient way [48, 49]. The concept of more efficient photosynthesis to increase yields has been considered by many researchers. Long et al. [50] identified the goals as improving the kinetic properties of RuBisCo, modifying C3 crops to confer the ability of C4 photosynthesis, and improving the canopy architecture. The classical approach is the change in stomatal conductance [51, 52].

In our studies, the NPP value directly depended on the treatment (Table 1). With Nanosilicon and the biological, the NPP was higher compared to both controls. Apparently, in the plants treated with these preparations, the contribution of assimilating organs was greater and the consumption of carbohydrates for respiration was less. Depending on the treatments, the value of NPP in spring wheat varied from 6.86±0.28 at the beginning of stem extension to 18.30±0.80 g/$m^2 \cdot$ day by the end of heading.

1. Net productivity of photosynthesis (g/$m^2 \cdot$ day) **in spring wheat (*Triticum aestivum* L., cv. Daria) plants depending on treatments and the stages of plant development** (n = 10, M±SEM; Streletskoe settlement, Orel Province, 2019 год)

Treatment	Stage				
	tillering	tillering—beginning of stem extension	tillering — beginning of heading	end of heading	beginning of ripening
Control (water)	0.49±0.02	6.86±0.28	7.70±0.35	10.10±0.37	5.00±0.24
Control (Vincite, KS)	1.39±0.06	7.90±0.34	8.80±0.28	9.50±0.33	6.11±0.45
Nanosilicon	1.40±0.07	8.70±0.41	14.20±0.55	18.30±0.80	10.33±0.80
Experimental biological	2.29±0.10	8.20±0.41	12.20±0.40	16.90±0.74	9.30±0.43

N o t e. For description of the treatments, see *Materials and methods.* The observed values for the Student's t-test exceed the tabulated values at df = 9 and P = 0.95, which indicates the reliability of the obtained differences in the net productivity of photosynthesis compared to the control values.

As is known, an increased leaf area in crops can reduce photosynthetic productivity, but this did not happen in our studies. The maximum leaf area at heading was 18000 m^2/ha which is far from optimal values [53].

The work of pigments that play a key role in photosynthesis depends on climatic and ecological factors [54, 55]. According to Andrianova et al. [35], the

amount of chlorophyll during tillering and stem extension may indicate the potential productivity of plants, since the formation of a high yield depends on the size of the assimilation apparatus and the time of its functioning.

In our experiments, the synthesis of pigments also reflected the effect of Nanosilicon on photosynthesis. The amount of pigments in leaves increased over the growing season by 20-70%, depending on the time of leaf functioning (Fig. 2, A). The amounts of chlorophyll and carotenoids were the greatest on July 13, that is, at the tillering—heading stage.

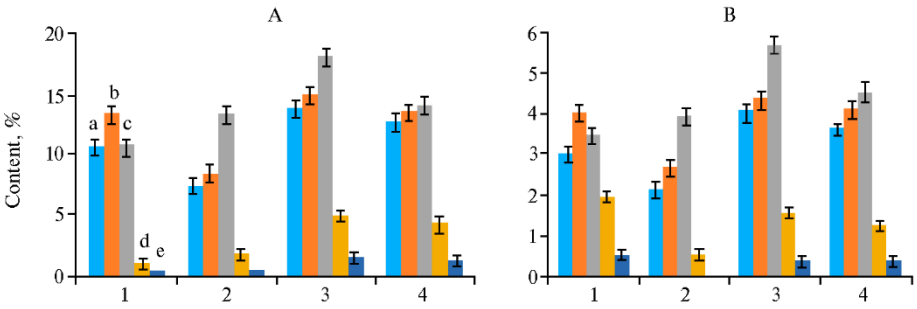

Fig. 2. Accumulation of chlorophylls a and b (A) **and carotenoids** (Б) **in spring wheat (*Triticum aestivum* L., cv. Daria) plants at tillering** (a)**, tillering—beginning of stem extension** (b)**, tillering—beginning of heading** (c)**, end of heading** (d)**, and beginning of ripening** (e) **stages as influenced by various treatments:** 1 — water, 2 — Vincite, KS desingectant, 3 — Nanosilicon, 4 — an experimental biological (n = 10, M±SEM; Streletskoe settlement, Orel Province, 2019).

In all experimental periods, plants treated with Nanosilicon and the biological exceeded the control in chlorophyll contents by 20-30%. Note, even during grain maturation, the leaves of these plants remained green which indicates a longer photosynthetic activity.

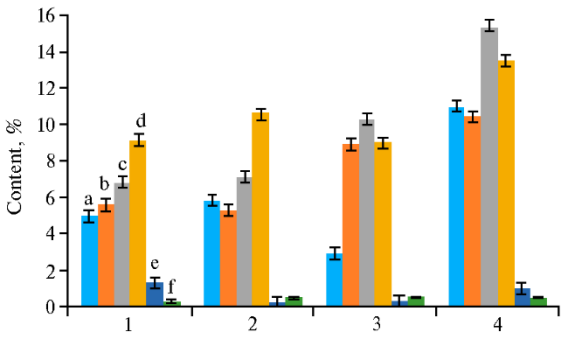

Fig. 3. Accumulation of sugars in spring wheat (*Triticum aestivum* L., cv. Daria) plants at plants at 2nd leaf (seedings) (a)**, tillering** (b)**, tillering—beginning of stem extension** (c)**, tillering—beginning of heading** (d)**, end of heading** (e)**, and beginning of ripening** (f) **stages as influenced by various treatments:** 1 — water, 2 — Vincite, KS desingectant, 3 — Nanosilicon, 4 — an experimental biological (n = 10, M±SEM; Streletskoe settlement, Orel Province, 2019).

Carotenoids absorb light in the blue-green region of the solar spectrum and transfer energy to chlorophylls, thereby expanding the wavelength range of light that can drive photosynthesis. They serve to increase the overall efficiency of photosynthetic light reactions and protect photosynthesizing organisms from overexposure to light [56]. The content of carotenoids often positively correlates with the amount of chlorophyll [57]. Our data confirm this conclusion (see Fig. 2, B). It is widely known that silicon significantly stimulates the growth of many plant species via increasing photosynthetic activity, leaf area, and chlorophyll content [58-62].

Nanosilicon enhanced sugar production to a lesser extent than the experimental biological, which can be explained by the redistribution of assimilates and, probably, a large accumulation of proteins in spring wheat under the influence of Nanosilicon (Fig. 3). Our hypothesis is in line with the data showing that mineral fertilizers significantly increase the mass fraction of protein and gluten in the grain

of spring wheat [63-65]. According to the report of Lavoy et al. [66], an increase in the protein content in wheat seeds under the influence of mineral fertilizers is due to a high correlation between the grain protein content and nitrate reductase activity, as is confirmed by Tao et al. [67]. The elevated grain sugar levels we observed under the influence of the experimental biological may have been associated with the level of salicylic acid which has a positive effect on the assimilation activity of plants [68, 69].

According to Mokronosov [70], growth regulators and phytohormones which contribute to a change in the outflow of assimilates from leaves to reproductive organs can ensure the greatest economic yield. Growth regulators and stimulants help to increase plant immunity, increase yields, and improve quality for obtaining environmentally friendly food [53].

We have found that the Nanosilicon application is an effective and low-cost technique to increase the overall yield of spring wheat. At the end of the growing season, the collected sheaves were heavier than in the controls and superior in the ear size. Under the influence of Nanosilicon, the number of productive stems increased by 33.7, of ears by 38.7, the weight of an ear by 26.8, the number of grains per ear by 19.2, and the 1000-grain weight by 19.7% compared to the control (water) (Table 2). For the biological, the indicators were slightly lower than for Nanosilicon but higher compared to both controls.

2. Yield components in spring wheat (*Triticum aestivum* L., cv. Daria) depending on treatments (n = 10, M±SEM; Streletskoe settlement, Orel Province, 2019 год)

Treatment	Productive stem number per 1 m^2	Ear		Grain number per ear	1000-grain weight, g	Ear number
		length, cm	weight, g			
Control (water)	326±11.2	8.2±0.37	1.9±0.08	32.8±0.84	37.7±1.49	300±11.6
Control (Vincite, KS)	276±10.2	8.3±0.31	1.9±0.08	33.1±1.47	40.1±1.94	244±10.6
Nanosilicon	436±17.3	9.0±0.43	2.4±0.11	39.6±1.63	45.1±2.00	416±16.9
Experimental biological	344±11.5	8.9±0.42	2.3±0.11	41.2±1.41	44.8±1.64	319±12.7

N o t e. For description of the treatments, see *Materials and methods*. The observed values for the Student's *t*-test exceed the tabulated values at df = 9 and P = 0.95, which indicates the reliability of the obtained differences compared to the control values.

3. Four-year yields of spring wheat (*Triticum aestivum* L., cv. Daria) depending on treatments (n = 10, M±SEM; Streletskoe settlement, Orel Province)

Treatment	Yield, c/hara				Gain to the control (water), %			
	2016	2017	2018	2019	2016	2017	2018	2019
Control (water)	28.9±1.38	38.2±1.61	41.3±2.04	45.1±2.00				
Control (Vincite, KS)	31.4±1.20	41.4±0.91	44.5±2.11	47.3±2.01	8.65	8.40	7.80	4.88
Nanosilicon	33.7±1.45	43.7±2.03	45.1±2.19	49.4±1.91	16.61	14.40	9.20	9.53
Experimental biological	34.5±1.55	43.9±2.07	45.3±1.71	48.9±2.16	19.01	14.90	9.20	8.43
LSD_{05}	1.2	1.4	1.2	0.9				

N o t e. For description of the treatments, see *Materials and methods*. The observed values for the Student's *t*-test exceed the tabulated values at df = 9 and P = 0.95, which indicates the reliability of the obtained differences compared to the control values.

Wheat yield under the influence of Nanosilicon increased due to an increase in both the grain number per ear and grain weight (Table 3). When treated with Vincite, KS, the yield increased by 8% compared to the control (water), with the exception of 2019 (only by 5%), and under the influence of Nanosilicon and the biological, its increase was 9-19%.

Thus, the Nanosilicon turned out to be more effective towards the spring wheat cv. Daria than the agrochemical Vincite, KS but differed little from the experimental biological product based on buckwheat bioflavonoids. Seed pre-treatment with Nanosilicon and the biological increased the germination energy by 18.5% and germination rate by 5.5% ($p < 0.05$) compared to the controls (water and Vincite, KS). The height of plants, the number and area of leaves, the photosynthetic potential, the indices of net productivity of photosynthesis (NPP), and

the amounts of chlorophylls and carotenoid indicate superiority of Nanosilicon over the controls. The NPP indices for Nanosilicon were 60-80% higher than the control (water) and 22.2% higher compared to agrochemical Vincite, KS. Under the influence of Nanosilicon and the biological, the synthesis of pigments increased by 20-30%. The biological product largely influences the synthesis of sugars than Nanosilicon, which can be explained by the difference in the redistribution of assimilates and the enhanced accumulation of proteins in plants under the influence of silicon. Nanosilicon provides an increase in the grain number per ear and the 1000-grain weight compared to both controls. The advantage over the bioactive proparation was insignificant. Nanosilicon increased the number of productive stems by 33.7, of ears by 38.7, the weight of an ear by 26.8, the grain number per ear by 19.2, and the 1000-grain weight by 19.7% compared to control ($p < 0.05$). Indicators for the biological product were slightly lower than for Nanosilicon but higher than the control. Wheat yield for four years under the influence of Nanosilicon and the biological increased by 9.0-16.6% as compared to control.

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STUDY OF THE BIOLOGICAL ACTIVITY OF ARABINOGALACTAN-STABILIZED SILVER NANOPARTICLES TOWARDS WATERCRESS *Lepidium sativum* L. cv. Curled AND PLANT PATHOGENIC MICROMYCETE *Fusarium sambucinum*

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Abstract

Metal nanoparticles (NPs) exhibiting growth-stimulating, antifungal, antibacterial, insecticidal effects and prolonged release of minerals and herbicides, opens up prospects for increasing the yield of crops. Among metal nanoparticles that can find application in agriculture, silver nanoparticles occupy a special place due to a wide spectrum of biological activity. In this work, we have established for the first time that the pre-sowing treatment of seeds of watercress *Lepidium sativum* L. cv. Curled by silver nanoparticles which are stabilized by the biopolymer arabinogalactan and dioctyl sulfosuccinate affects the germinative energy, laboratory seed germination and some anatomical and morphometric parameters of watercress seedlings. It was shown for the first time that silver nanoparticles have an inhibitory effect on the growth of the phytopathogenic fungus *Fusarium sambucinum*. This work aimed to assess both the stimulating effect of silver nanoparticles (Ag-NPs) stabilized with arabinogalactan and dioctyl sulfosuccinate on growth of watercress *Lepidium sativum* L. cv. Curled seedlings and the antifungal effect on a plant pathogenic toxin-producing micromycete *Fusarium sambucinum* VKPM F-900. The nanoparticles were synthesized by the reduction of silver nitrate in an alkaline medium in the presence of arabinogalactan followed by the addition of dioctyl sulfosuccinate as a stabilizer. The average nanoparticle diameter was 11.40±3.96 nm; zeta potential –24 mV. The effect of silver nanoparticles on germination energy, seed germination, growth of watercress seedling hypocotyl and root was investigated. Seeds were incubated in sols of nanoparticles with various silver concentrations (1.17, 2.34, 4.69, 9.38, 18.75. 37.5, 75, and 150 μg/ml). Control seeds were incubated in water. After incubation, the seeds were germinated in Petri dishes on a wet bed of filter paper in the dark at 20 °C. The seed germination energy was determined on day 3, the laboratory germination — on day 5, the lengths of the hypocotyl and the main root were measured on day 7, and also microscopic analysis of the root sections of seedling treated with sols with stimulating and inhibiting concentrations of Ag (4.69 and 18.75 μg/ml, respectively) was carried out. Antifungal activity of silver sols with concentrations from 9.38 to 300 μg/ml was assessed by the agar diffusion method. Micromycete *Fusarium sambucinum* Fuckel VKPM F-900 was used as a test culture to determine antifungal activity. Sterile water was used as a control. The incubation of seeds in sols with a silver concentration of 2.34 and 4.69 μg/ml had a stimulating effect on the germination energy and laboratory germination of *L. sativum* seeds. A dose of silver nanoparticles of 4.69 μg/ml increased the germination energy by 13.5 % and laboratory germination by 11.7 % compared to the control. In addition, the concentrations of silver from 1.17 to 4.69 μg/ml had a significant stimulating effect on root growth (from 34.4 to 79.1 %, respectively) with some deceleration of hypocotyl growth. Seed incubation in sols with a silver concentration of 18.75 μg/ml and higher led to a significant decrease in the germination energy and laboratory germination, as well as suppression of plant growth. Microscopic examination of sections of zone of maturation of the root of seedlings showed that silver sols significantly affect the conductive system of the central

axial cylinder. The number of xylem vessels in seedlings treated with silver sol at a stimulating concentration of 4.69 μg/ml was significantly higher compared to the control, which led to a more intensive growth of the root system and the whole plant. Silver nanoparticles also inhibit the growth of *F. sambucinum*. The growth inhibition zone at a maximum sol concentration of 300 μg/ml was 32.4±4.2 mm in diameter, and at 150 μg/ml it was 28.4±3.9 mm. The minimum concentration inhibiting the visible growth of the test strain *F. sambucinum* was 18.75 μg/ml (growth inhibition zone 11.7±0.8 mm). The presented data indicate the possibility of using sols of stabilized silver nanoparticles to stimulate seed germination and plant growth and to protect plants against pathogens.

Keywords: silver nanoparticles, plant growth, germinative energy, seed germination, antifungal activity, *Lepidium sativum*, *Fusarium sambucinum*

Biotechnologies and nanotechnologies in plant farming are promising due to the wide opportunities to increase crop yields by increasing plant resistance diseases, pestsn and adverse environmental factors [1]. The use of nanoparticles (NPs) with a growth-stimulating effect, antifungal, antibacterial, insecticidal activity, and prolonged release of minerals and herbicides opens up prospects for increasing crop productivity and a better storage of seeds and food products to overcome food shortages [2]. Among metals, silver nanoparticles (Ag NPs) occupy a special place due to a wide spectrum of biological activity [3, 4].

The Ag NPs are of interest as a tool to stimulate plant growth and protection [5-7]. Ag NPs, especially at high concentrations, are able to penetrate into plant tissues and accumulate [6, 8, 9]. However, NPs can have both a stimulating and an inhibitory effect on plant growth [10]. Thereof, a comprehensive understanding the NPs suitability for practical use and mechanisms of their action on a plant requires additional studies. An important area of Ag NPs application is plant protection from pathogenic fungi which significantly reduce productivity and cause contamination of animal feeds and agricultural products with hazardous mycotoxins [11]. The fungicidal properties of Ag NPs against plant fungal pathogens *Rhizoctonia solani*, *Fusarium semitectum* [12-14], *Bipolaris sorokiniana*, *Magnaporthe grisea* [15, 16], *Alternaria solani*, *Pythium spinosum*, *Pythium aphanidermatum*, *Cylindrocarcupon destructum*, *Didymella bryoniae*, *Stemphylium lycopersici*, and *Monosporascus cannonballus* [17], however, the potential of the antifungal activity of Ag NPs has not been fully dislosed.

The use of Ag NPs in agriculture necessitates reliable, efficient, and inexpensive methods for the synthesis of nanoparticles [18, 19]. Environmentally friendly reducing agents with low toxicity to humans and stabilizers of natural origin are most preferable, e.g., various polymers [20, 21] and arabinogalactan, a polysaccharide from Siberian leaf tree (*Larix sibirica*) and garden purslane (*Portulaca oleracea*) [22, 23]. Due to water solubility, thermal stability, biocompatibility, and the possibility of using it as a reducing agent and stabilizer in the synthesis of Ag NPs, arabinogalactan is recognized a promising biopolymer for the development of nanostructures and nanocomposites for agriculture [24, 25].

Reduction from silver nitrate by the so-called “green synthesis” method using fungal mycelium [26] and plant extracts [27, 28] is a safe and efficient technique to produce Ag NPs. Despite the attractiveness of the reduction method, the use of arabinogalactan seems to be more preferable, since, in addition to biosafety, biogenic synthesis occurs under more controlled conditions, and, therefore, nanoparticles have properties that are more predictable.

In this work, we have established for the first time that the pre-treatment of cress (*Lepidium sativum* L., cv. Curled) seeds with silver nanoparticles stabilized with the biopolymer arabinogalactan and dioctyl sulfosuccinate affects the germination energy, germination rate and some anatomical and morphometric parameters of seedlings. Our findings have shown for the first time an inhibitory effect of Ag NPs on the growth of the pathogenic fungus *Fusarium sambucinum*.

The aim of this work was to assess the potential of silver nanoparticles stabilized with arabinogalactan and dioctyl sulfosuccinate as a stimulant of the growth of Curled cress and an antifungal agent against mycotoxin-forming micromycete *Fusarium sambucinum* VKPM F-900.

Materials and methods. For silver nanoparticle synthesis, we used silver nitrate (JSC LenReaktiv, Russia), ammonium hydroxide (27%), sodium dioctyl sulfosuccinate Aerosol-OT, or bis(2-ethylhexyl) sulfosuccinate, sodium salt (Labtex, Russia), and arabinogalactan (Fluka, Germany). A solution of silver nitrate was added to a 0.2% solution of arabinogalactan heated to 90 °C with vigorous stirring. The reduction reaction was carried out for 40 min at the same temperature and pH ≥ 10.0, followed by the addition of sodium dioctyl sulfosuccinate to a final concentration of 0.8% and gradual cooling to room temperature. Electrokinetic potential of silver nanoparticles was determined on a Photocor compact Z analyzer (OOO Photocor, Russia). Transmission electron microscopy was performed using a LEO 912 AB microscope (Carl Zeiss, Germany) at an accelerating voltage of 100 kV. To prepare the samples, a drop of sol was applied to copper grids with a diameter of 3.05 mm, covered with a thin polymer film, and dried at room temperature. The size distribution of Ag NPs was determined by processing the obtained micrographs using ImageTool 3.00 software for the analysis of optical images (UTHSCSA, USA).

The effect of Ag NPs on germination energy, seed germination, hypocotyl and root growth of cress cv. Curled was evaluated in Petri dishes. The seeds were incubated for 1 h in sols of nanoparticles with different silver concentrations. i.e., 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75, and 150 μg/ml (three replicates of 100 seeds per each treatment). Control seeds were incubated in water. After the end of the incubation, the seeds were germinated in Petri dishes on a wet filter paper in the dark at 20 °C. Germination energy was measured on day 3, the germination rate on day 5, the length of the hypocotyl and the main root of the seedlings on day 7.

The morphology of *L. sativum* cells was examined microscopically using root sections of 7-day-old seedlings treated with sols with stimulating and inhibiting Ag concentrations (4.69 and 18.75 μg/ml, respectively). Seedlings not treated with sol of Ag nanoparticles served as control. In the zone of seedling roots, 100-150-μm thick unfixed cross sections were prepared manually, without preliminary or subsequent fixation. The sample for each treatment consisted of 30 plants (10 from each of three independent experiments). Sections were embedded in water:glycerol (1:1) liquid and examined under a light microscope LOMO Mikmed-6 (LOMO JSC, Russia) at ×100 and ×400 magnifications. Images were obtained using a digital camera attachment Canon Digital IXUS 80 IS (Canon, Japan) and processed using the Microsoft Office Picture Manager program. The quantitative processing of photographs (n = 10 for each experimental group) was carried out using the CellProfiler program (https://cellprofiler.org) [29].

The antifungal activity of Ag nanoparticle sols was assessed by the agar diffusion method [30]. Micromycete *Fusarium sambucinum* Fuckel VKPM F-900 cultured on agar Saburo medium (PanEko, Russia) was a test culture. In wells 10 mm in diameter, 400 μl of Ag sols were added (concentrations of 9.38, 18.75, 37.5, 75, 150, and 300 μg/ml, prepared by double dilutions; control was sterile water). The experiment was carried out in three replicates, 4 wells for each dose. Petri dishes with the test strain were incubated for 5 days at 27 °C and examined for the presence of zones of growth inhibition around the wells.

The results were statistically processed using Microcal Origin 8.0 software (OriginLab Corporation, USA). All data in tables and figures are arithmetic mean values (M) and standard deviations (±SD). To identify the statistical significance of the differences, one-way analysis of variance (ANOVA) was used; differences

were considered significant at $p < 0.05$.

Results. The Ag sol was obtained by the reduction of silver nitrate using arabinogalactan, which simultaneously acted as both a reducing agent and a stabilizer for nanoparticles. To increase the stability of the Ag sol, sodium dioctyl sulfosuccinate was added. According to transmission electron microscopy data, the preparation contained spherical Ag NPs (Fig. 1, A). The average calculated diameter of the nanoparticles was 11.40±3.96 nm (see Fig. 1, B), zeta potential –24 mV.

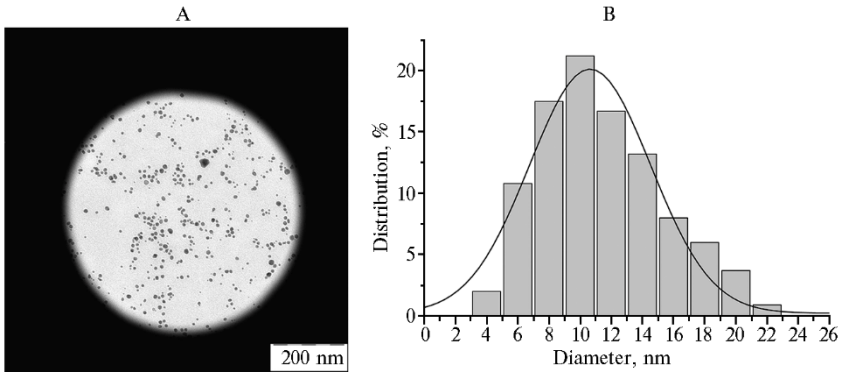

Fig. 1. Shape and size of Ag nanoparticles stabilized by arabinogalactan and Na-dioctyl sulfosuccinate: A — Ag sol (transmission electron microscopy, LEO 912 AB, Carl Zeiss, Germany; ×1500 magnification), B — distribution of nanoparticles by size (UTHSCSA ImageTool 3.00).

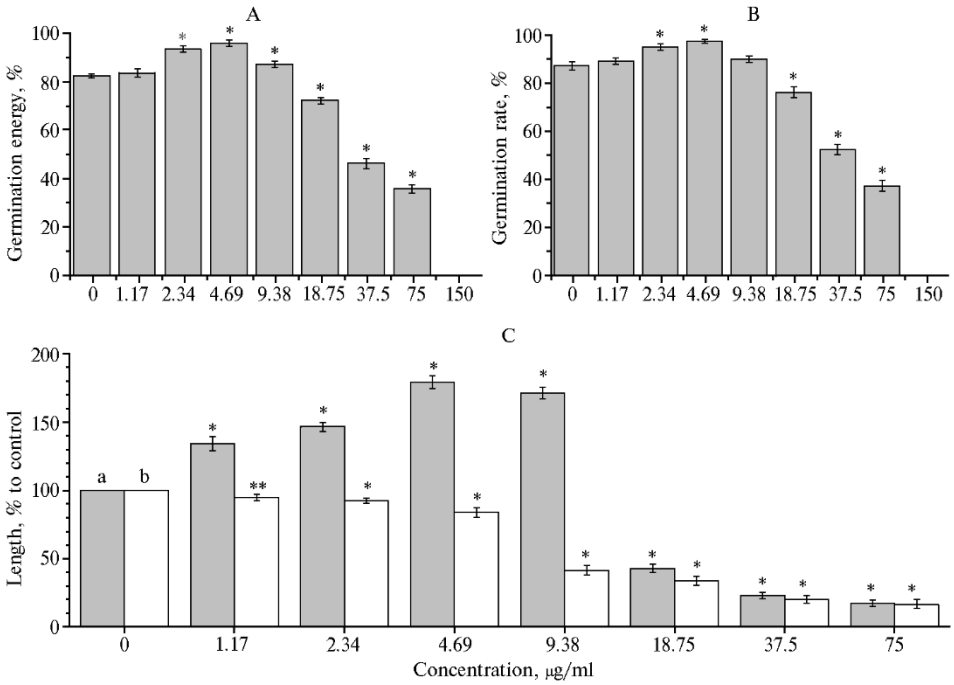

Fig. 2. Germination energy (A)**, germination rate** (B)**, the length of the main root** (a, C) **and hypocotyl of seedlings** (b, C) **in cress (*Lepidium sativum* L., cv. Curled) upon treatment with sols of nanoparticles with different concentrations of Ag** ($n = 10$, M±SD)**.**
*, ** Differences from control are statistically significant at $p < 0.01$ and $p < 0.05$, respectively.

On day 3, the germination energy in the control was 83.00±0.82% (Fig. 2, A). At the lowest Ag concentration (1.17 μg/ml), no stimulation occurred (the indicators did not differ statistically from the control). At 2.34 and 4.69 μg/ml, a pronounced stimulating effect occurred. At 4.69 μg/ml, it reached a maximum of 96.5±1.29% (13.5% higher than in the control, $p = 2.1 \times 10^{-6}$). Seeds exposed to

sols with high Ag concentrations (9.38-150 μg/ml) exhibited dose-dependent inhibitory effects. The Ag concentration of 150 μg/ml showed the maximum inhibitory effect, and the seeds did not germinate.

Seed germination rate was determined on day 5. The seeds exposed to Ag concentration of 1.17 μ`g/ml, showed no noticeable effect ($p = 0.11$) (see Fig. 2, B). At 2.34 and 4.69 μg/ml, the stimulation occurred, with an 8.8 and 11.7% increase in germination rate compared to the control ($p = 3.5\times10^{-4}$ and $p = 3.7\times10^{-5}$). The germination capacity of seeds incubated in 9.38 μg/ml Ag sol was comparable to the control ($p = 0.04$). At higher Ag concentrations, this indicator consistently decreased and was significantly lower than in the control ($p < 0.01$).

On day 7, at 1.17, 2.34, and 4.69 μg/ml Ag, the development of hypocotyl slightly slowed down while a significant dose-dependent root stimulation occurred. The length of the root significantly exceeded the control (by 34.4%, $p = 8.9\times10^{-4}$; 46.4%, $p = 8.2\times10^{-5}$ and 79.1%, $p = 6.3\times10^{-5}$). At 9.38 μg/ml Ag in sol, the length of the hypocotyl was significantly (by 58.7%, $p = 5.8\times10$ 5) less and the length of the root was 71.3% higher than in the control ($p = 5.8\times10$ 5), which indicates a significant stimulating effect of Ag NPs on root growth while suppressing hypocotyl growth. Upon incubation of seeds in sols with higher Ag concentrations (18.75, 37.5, and 75 μg/ml), the inhibitory effect consistently increased towards both hypocotyl and root ($p < 0.01$) (see Fig. 2, C).

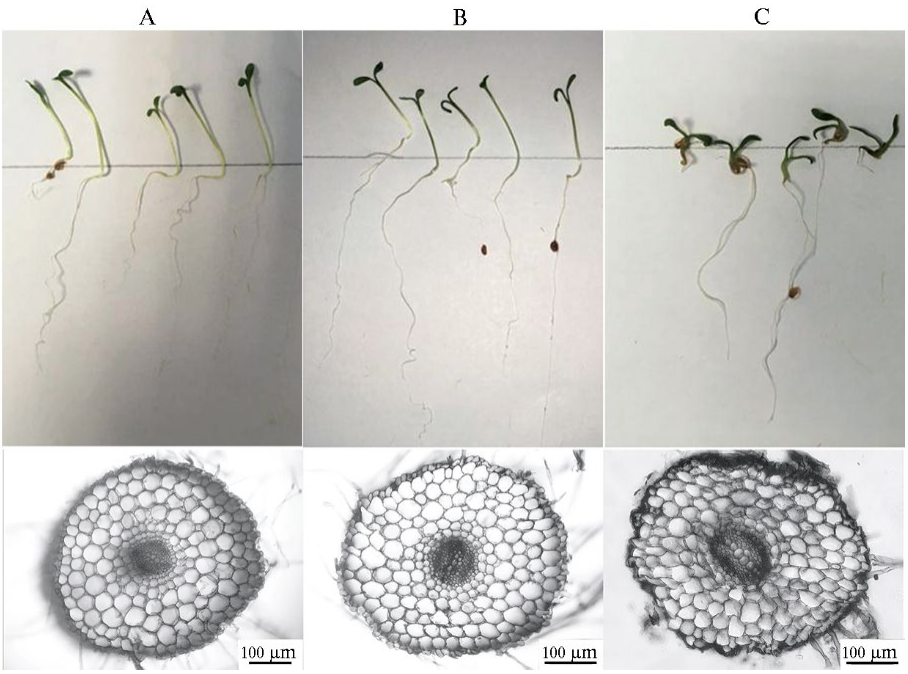

Fig. 3. Seedlings of cress (*Lepidium sativum* L., cv. Curled) 7 days after seed germination in control (A) **and upon seed exposure to Ag sols with concentrations 4.69** (B) **and 18.75 μg/ml** (C)**:** at the top — general view; below — photomicrographs of root cross sections (light microscope LOMO Mikmed-6, Russia, magnification ×400).

Root sections both in control (without seed pre-treatment) and upon seed treatment with Ag sols showed a characteristic anatomical picture of the primary root structure (Fig. 3). The integumentary tissue (a single-layer epiblema), the primary cortex, consisting of 3-4 layers of the mesoderm and one inner layer of the endoderm, and the central axial cylinder of the diarchic structure were distinguishable. The cells of the mesodermal parenchyma were round-oval in shape with more or less pronounced rectangular intercellular spaces.

Anatomic structure of roots in seedings of cress (*Lepidium sativum* L., cv. Curled) upon treatment with sols of nanoparticles with different concentrations of Ag (n = 3, M±SD)

Ag concentration, μg/ml	Number of vessels.	Diameter of parenchyma cells, μm
Control (no treatment)	12.5±3.8	58.3±12.7
4.69	21.5±5.6*	34.2±5.9*
18.75	13.9±4.1	35.6±9.5*

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

Comparing the root anatomical structure of the control and test seedlings, we revealed the Ag sol at a stimulating concentration of 4.69 μg/ml to provide the formation of polygonal parenchymal cells with a 41.3±12.4% decrease in size compered to control (Table). The intercellular spaces of the primary cortex parenchyma tended to decrease and practically disappeared at the inhibitory concentration (18.75 μg/ml). Inhibitory concentration also affected epiblemal cells and peripheral layers of mesoderm cells, causing thickening of cell walls and a 38.9±8.8% decrease ($p < 0.05$) in the size of parenchymal cells compared to control (see Table). Ag NPs had a significant effect on the conducting system of the central axial cylinder. The number of xylem vessels in the roots upon treatment with a stimulating Ag concentration was significantly higher ($p < 0.05$) compared to the control and an inhibitory concentration (see Table).

The size of nanoparticles directly influences not only their physicochemical properties, but also biological activity [31]. The smaller the NPs are, the higher their specific surface area and activity in overcoming physical and biological barriers. In our work, we used Ag NPs of very small size (11.40±3.96 nm) stabilized with arabinogalactan and dioctyl sulfosuccinate, which may be one of the important reasons for their active penetration through the seed coat and stimulation of germination energy, germination rate, and seedling growth. Obviously, stimulation can occur at moderately low concentrations (2.34 and 4.69 μg/ml), since higher concentrations inhibit plant growth as a result of damaging effects on biomembranes, macromolecules and disruption of the integrity of cell organelles. This conclusion is consistent with the data of Kaveh et al. [32]. The sol of high Ag NPs concentration (18.75 μg/ml), on the contrary, inhibits the formation of conductive elements in roots, suppresses root growth, and retards plant development.

The positive effect of nanoparticles on plant growth may be associated with the stimulation of photosynthetic processes, as well as with the activation of the auxin synthesis system in meristematic tissues [18]. Our study of root morphology showed that the action of 4.69 μg/ml Ag NPs led to the formation of numerous conductive elements, which was one of the important reasons for the activation of growth both roots and the entire plant due to an improved supply of water and minerals. The effect of dose-dependent stimulation of root growth that we revealed is consistent with the data of Geisler-Lee et al. [33] on accumulation of silver nanoparticles in the root cap in seedlings of *Arabidopsis thaliana*. It is obvious that the pattern of NPs distribution in a plant affects the predominant growth of certain tissues. However, the mechanism of the action has not yet been disclosed.

In this work, we also established the ability of stabilized metallic silver nanoparticles to suppress the growth of the pathogenic fungus *F. sambucinum*. Fungi of the genus *Fusarium* are mold pant pathogens that produce mycotoxins hazardous to human health [11]. Since the sources of mycotoxins can be infected green plants, animal feed, and food, an important task is to find new and expand the range of available means of combating mold fungi.

Sols had a dose-dependent inhibitory effect on the growth of *F. sambucinum* VKPM F-900. At 9.38 μg/ml concentration, we did not observe inhibition of fungal growth. The minimum concentration which inhibited the visible growth of the *F. sambucinum* test strain, was 18.75 μg/ml (inhibition zone of 11.7±0.8 mm). At 37.5, 75, 150, and 300 μg/ml, the diameters of the growth inhibition zone were 12.9±0.9, 21.1±2.7, 28.4±3.9, and 32.4±4.2 mm. The antifungal effect may be due to damage to the cell wall and phospholipid membranes by Ag nanoparticles, as well as to the disruption of the respiratory chain and the nuclear DNA of the fungus caused by silver ions dissociating from nanoparticles in biological media [34]. Inhibition of the growth of *F. sambucinum* allows us to consider the drug as a potential means of increasing yields and protecting plants and agricultural products from mold fungi and mycotoxins hazardous to human health.

Thus, in *Lepidium sativum* L. cv. Curled, the exposition of seeds to sols of nanoparticles (NPs) with a silver concentration of 2.34 and 4.69 μg/ml has a stimulating effect on the germination energy, germination rate and growth of the root of seedlings while slightly decelerates the hypocotyl growth. Ag NPs at a concentration of 4.69 μg/ml stimulate the development of the root conducting system in the seedlings. Sols with Ag concentrations exceeding 4.69 μg/ml exhibit toxic effects and inhibit plant growth. Exposure to sols of Ag NPs with a concentration of 18.75 μg/ml and higher leads to a significant decrease in the energy of germination, germination rate and suppresses plant growth. Ag NPs sols also inhibit plant pathogen *Fusarium sambucinum* (the minimum inhibitory concentration is 18.75 μg/ml). Therefore, silver sols stabilized with arabinogalactan and dioctyl sulfosuccinate have a significant potential as plant stimulants which can also protect from plant pathogens. A better understanding of advantages and obstacles of using stabilized silver sols in agriculture requires additional in-deep studies.

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BIOLOGICAL BACKGROUND TO USING CHITOSAN INDUCERS TO INCREASE THE EFFICIENCY OF BIOFUNGICIDES

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Abstract

Microbiological preparations form the basis of modern technologies phytosanitary optimization of agroecosystems, therefore, increasing their efficiency in protecting crops from a wide range of plant pathogens is an urgent task of crop production. The All-Russian Research Institute of Plant Protection (VIZR) has developed Vitaplan, a biological product based on the composition of two strains, the *Bacillu subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D with a different composition of active complexes and a mechanism of action that are highly effective against a wide range of plant pathogens. The aim of the study is to substantiate increasing the biological effectiveness of new Vitaplan formulations supplemented with chitosan as an inducers of plant resistance. In this work, for the first time, two new formulations were developed, the Vitaplan, CF + colloidal chitin and the Vitaplan, CF + 0.1 % chitosan salicylate with increased antagonistic and elicitor activity compared to the original biological Vitaplan, CF. The effect of disease resistance inducers, such as colloidal chitin and chitosan salicylate, on the biological activity of Vitaplan has been investigated. The colloidal chitin (1 %) added to the deep culture medium increased the antagonistic activity of *B. subtilis* VKM B-2604D and VKM B-2605D against the tomato bacterium *Clavibacter michiganensis* subsp. Michiganensis (Smith) Davis et al. (strain 101) and the fungus *Alternaria solani* Sorauer, and also ensured effective suppression of the growth of *Cochliobolus sativus* mycelium up to 84.9-88.1 % on day 5 and day 7 of the experiment, which is comparable to the efficiency of Vitaplan CF (80.9-87.5 %, respectively). Chitosan salicylate at a concentration of 0.1 % had a moderate fungistatic activity, with only 36.5-46.0 % suppression of the growth of the *C. sativus* mycelium. The study of the immunomodulatory activity of various Vitaplan CF preparative forms in protecting wheat against the brown spot pathogen *Cochliobolus sativus* (S. Ito & Kurib.) was carried out under various infectious loads. Depending on the infectious load of the pathogen, preliminary spraying of wheat plants with Vitaplan, CF at a dilution of 1:10 followed by infection with the brown spot pathogen reduced the leaf lesion area to 50-80 % compared to 65-100 % in control. Adding 1.0 % colloidal chitin to the culture medium for the producer strains reduced leaf damage to 40-50 %, which indicates a higher immunomodulatory activity of this form of Vitaplan compared to Vitaplan CF at a dilution of 1:10. Chitosan salicylate proved to be an effective inducer of disease resistance, reducing damage to wheat plants by dark brown spots to 10-20 %, depending on the infectious load. The addition of chitosan salicylate at a concentration of 0.1 % to original form Vitaplan, CF at a 1:10 dilution also had a positive effect on the antagonist with a 2.0-2.5-fold increase of its biological activity. The biological effectiveness of the new formulation Vitaplan CF (1:10) + chitosan salicylate (0.1 %) in protecting wheat from brown spot when spraying plants is determined by two mechanisms, i.e., by i) direct pathogen suppression due to antibiotics and enzymes the *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D produce, and ii) through the induction of plant systemic resistance. The chitosan salicylate in the Vitaplan biological increases the inducing activity of this new formulation compared to the original form. Thus, the prospect of combining active selected strains of microbial antagonists of plant pathogens and chitosan complexes to increase the biological efficiency and expand the spectrum of action of drugs has been experimentally confirmed and theoretically substantiated.

Keywords: biological control, *Bacillus subtilis*, Vitaplan formulations, fungistatic activity, antagonistic effect, chitosan, chitin, systemic resistance, *Triticum aestivum* L., *Cochliobolus sativus*

The widespread use of plant protection chemicals and mineral fertilizers formed the base of traditional intensive farming in the 20th century. However, having increased the yield, intensive technologies have led to a phytosanitary deterioration of crops due to appearance of pesticide-resistant populations of pest species, a decrease in product quality, soil degradation and a drop in soil fertility. In this regard, the development of new biological means of protecting agricultural crops from harmful organisms is relevant. Environmentally friendly crop production should ensure a decrease in the chemical load on agroeco-systems, optimization of soil microbial community and restoration of microbiological soil activity.

Microbiological preparations are the main element of technologies for phytosanitary optimization of agroecosystems with relevance to biology. According to numerous studies, bacteria of the genus *Bacillus* Cohn. are one of the most promising for biological control of plant pathogens [1-4].

Biologicals based on *B. subtilis* strains are efficient against plant diseases on the main agricultural crops in Russia and abroad. Bacillus strains suppressed the spread and development of fusarium wilting of maize [5], fusarium ear of wheat [6, 7], fusarium and ophiobolous root rot [8], powdery mildew [9], yellow and brown rust of cereals [10-12]. Biopreparations based on this group of antagonist bacteria significantly reduce the incidence of leaf diseases in rice [13]. In vegetable crops, *B. subtilis* strains are efficient against fusarium and bacteriotic wilting of tomato [14, 15], cucumber root rot [16], and phytophluorosis of pepper [17]. *Bacillus*-based preparations s protect strawberries from rust [18]. A number of reviews focused on obstacles and prospects for the use of *B. subtilis*-based biologicals [19-21].

The effectiveness of controlling the density of populations of plant pathogens when using microbiological plant protection products depends on the biological characteristics of producer strains and is due to a number of factors. These include both successful competition for nutrients and space for soil and rhizosphere colonization [22-23], and the ability of microorganisms to synthesize bioactive compounds (antibiotics, biosurfactants, siderophores, etc.) [24-26]. Production of hydrolytic enzymes (chitinases, glucanases, proteases, and lipases) which destroy the cell walls of plant pathogenic fungi, is of great importance [26]. In addition to direct antagonistic action on the cells of the causative agent, bacilli are able to increase plant disease resistance due to the presence of bacterial determinants (microbe-associated molecular patterns, MAMPs), such as flagellin, lipopolysaccharides (LPS), and other compounds associated with the cell wall of *B. subtilis* [27-29], as well as volatile organic substances [30]. The synthesis of elicitor compounds activates induced resistance of plants. Elicitors induce a nonspecific immune response, but also stimulate the production of plan hormones, i.e., salicylic acid (activator of systemic induced resistance) and jasmonic acid (activator of systemic acquired resistance).

Bacillus strains used for biologicals are capable of a variety of metabolic processes, including production of bioactive substances (BAS) which differ in their chemical nature and mechanisms of action. In this regard, it is very promising to design compositions from different strains or species of microorganisms to provide high activity of various biologicals.

A biological product Vitaplan (developed at the All-Russian Research Institute of Plant Protection. VIZR) is based on two strains, the *B. subtilis* VKM V-2604D and *B. subtilis* VKM V-2605D with different bioactive complexes and mechanisms of action which are highly effective towards a wide range of plant pathogens [4, 31].

Enhancing the ability of bacteria to trigger a cascade of defense reactions and increase the systemic resistance of plants is a way to increase the effect of

biologicals. Natural or synthetic activators of disease resistance in the formulation can strengthen the inducing activity of the producer strain. Natural inducers, e.g., polysaccharides (chitin, chitosan) and salicylic acid (SA) as a signaling molecule of systemic acquired resistance are most preferable. Chitosan and chitosan-based preparations are common in plant protection against diseases as inducers of non-specific resistance [32-34], and SA is a classical inducer of disease resistance which plays a central role in protecting plants from biotrophic pathogens [35].

Biological product Vitaplan is included in the State catalog of pesticides and agrochemicals permitted for use on the territory of the Russian Federation in the form of a wettable powder (WP). When developing new multifunctional formulations, we used the previously obtained data on the high immunomodulatory activity of the conjugate of chitosan with SA (chitosan salicylate) [36].

In this work, for the first time, we have developed two new optimized formulations of Vitaplan, KZh (Vitaplan, KZh + colloidal chitin and Vitaplan, KZh + 0.1% chitosan salicylate) with increased antagonistic and elicitor activity compared to the original form of the biological product. It was found that the chitosan salicylate in the new formulation Vitaplan, KZh increases the inducing activity by 2.0-2.5 times compared to the original formulation.

The purpose of our research was to improve the effectiveness of the multifunctional biological Vitaplan by including chitosan-based disease resistance inducers in the formulation.

Materials and methods. The strains *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D (State collection of microorganisms pathogenic for plants and their pests, Center for collective use of scientific equipment "Innovative technologies for plant protection" VIZR; registered January 28, 1998 at No. 760 in the World Federation for Culture Collections, World Data Center for Microorganisms — WFCC WDCM, Japan) were used. Bacteria were cultured on an artificial nutrient medium (30 g/l corn extract, 15 g/l molasses, pH 7.2; a laboratory shaker, 28 °C, 220 rpm for 72 h, 750 ml flasks, and 100 ml medium volume).

The antagonistic activity against *Alternaria solani* Sorauer and *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davisetal. (strain 101) was assessed for i) Vitaplan, KZh (titer of viable cells 10^{10} CFU/ml), the microbial culture of *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D strains at a 1:1 ratio without additives (control); ii) Vitaplan, KZh (10^{10} CFU/ml) + 1.0% dry chitin; iii) Vitaplan, KZh (10^{10} CFU/ml.) + 1.0% colloidal chitin as calculated per the dry weight of chitin; and iv) Vitaplan, KZh (1010 CFU/ml) + 1.0% colloidal chitosan as calculated per the dry weight of chitosan.

Fungistatic activity against *Cochliobolus sativus* S. Ito & Kurib. Drechsler ex Dastur (=*Bipolaris sorokiniana*, =*Drechslera sorokiniana* Subram et Jain, =*Helminthosporium sativum* Pam.) was evaluated as follows: i) control (water); ii) Vitaplan, KZh (10^{10} CFU/ml); iii) Vitaplan, KZh (diluted 1:10 with distilled water, 10^9 CFU/ml); iv) Vitaplan, KZh (without dilution, 10^{10} CFU/ml) + 1.0% colloidal chitin as calculated per the dry weight of chitin; v) Vitaplan, KZh (diluted 1:10 with distilled water, 10^9 CFU/ml) + 0.1% chitosan salicylate; and vi) 0.1% chitosan salicylate.

To study the inducing activity in the pathosystem of wheat (*Triticum aestivum* L.)—*C. sativus*, the Vitaplan, KZh; Vitaplan KZh + 1% colloidal chitin; and Vitaplan, KZh + 0.1% chitosan salicylate were diluted 10 times with distilled water (as per the norms of using Vitaplan KZh), the titers of all working solutions were 10^9 CFU/ml. The 0.1% chitosan salicylate was used.

For colloidal chitin, 100 kDa chitin was dissolved in concentrated hydrochloric acid followed by precipitation with acetone [38]. For colloidal chitosan,

the method developed by us was applied. Dry chitosan (100 kDa. 1 g) was dissolved with permanent stirring in 100 ml of a 2.5% aqueous solution of lactic acid and neutralized with 1.5% aqueous sodium hydroxide to pH 8.0. The colloidal solution of chitosan was kept in a refrigerator to form a precipitate.

Chitosan (60 kDa) was obtained by the oxidative destruction method [37] from 150 kDa chitosan with 85% deacetylation (Bioprogress, Russia) to synthetize chitosan salicylate containing 25% of ion-bound SA fragments. The bands from the CO_2^- carboxylate group in the IR spectrum (1552.92 cm^{-1} and 1386.12 cm^{-1}) were characteristic of a salt between chitosan and SA. A broad strong band at 3100-2600 cm^{-1} corresponded to stretching vibrations from the NH_3^+ and OH^- functional groups.

The titer of viable cells in the samples was determined by the 10-fold serial dilutions with plating on SPA agar medium and counting colonies. Antibacterial activity against the causative agent of bacterial canker of tomato *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davisetal. (strain 101) and antifungal activity against *Alternaria solani* Sorauer was assessed by the paper disk method by the diameter of the lysis zone of the test pathogens on agar nutrient medium. Czapek agar in Petri dishes was plated with 10^5 CFU/ml suspension of the test culture and placed on the agar surface sterile paper filters (diameter 8 mm) onto which a suspension of a certain concentration of a test formulation was applied with a pipette. Test cultures were grown in a TC-1/80 SPU thermostat (SKTB SPU, Russia) at 22-25 °C for 3-5 days.

The direct fungistatic effect of the studied formulations was assessed in vitro by the method of agar blocks. Czapek agar medium cooled to 40 °C was poured into sterile Petri dishes. After solidification, suspensions of the test formulations (0.2 ml) were evenly applied to the surface of the agar medium. Blocks (6 mm diameter) of 10-day-old cultures of *C. sativus* cut with sterile drill from the 8-10-day mycelial culture on Czapek agar were also placed on the agar medium. Plates with Czapek agar medium with blocks of test culture without specimens of the formulations served as a control. The dishes were incubated in the dark at 25 °C. The diameters of the fungus colonies were measured on days 5 and 7 of co-culture, and the fungistatic effect of the test samples was assessed as per the Abbott formula: $S = (D_c - D_{test})/D_c \times 100\%$, where S is the suppression of the fungal growth compared to control, %; D_c is the diameter of the fungus colony in the control, mm; D_{test} is the diameter of the colony of the fungus in the test treatment, mm.

Experiments to assess the immunomodulatory activity of samples of preparative forms were carried out by the method of detached leaves [39]. Twenty-four hours before inoculation with the pathogen, 7-day-old wheat seedlings of the disease-susceptible cultivar Saratovskaya 29 were sprayed with suspensions of the formulations diluted 1:10. Wheat leaves were infected with a spore suspension of *C. sativus* (4×10^3 and 20×10^3 spores/ml). The degree of leaf infestation was assessed on day 4 as a percentage of the affected leaf area. Control plants were treated with water.

All experiments were performed in three replicates. An analysis of variance (the Statistica 6.0, StatSoft, Inc., USA and Excel 2016 programs) was used to process the data. In the calculations, the methods of parametric statistics based on mean values (*M*) and standard errors of means (±SEM), 95 % confidence intervals, the least significant difference at $p < 0.05$ (LSD_{05}) were applied.

Results. Chitin and chitosan in the medium for submerged cultures of *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D had no negative effect on the cell titer of these strains use to produce the Vitaplan, KZh biological (Table 1). The colloidal chitin in the medium slightly increased the antagonistic activity

of the producer strains towards both test cultures.

Vitaplan, KZh showed high fungistatic properties which did not differ significantly for 10^9 and 10^{10} CFU/ml (74.3 and 80.9% suppression the growth of *C. sativus* mycelium on day 5, and 81.2 and 87.5% suppression on day 7). With the addition of 1.0% colloidal chitin to the growth medium, the biological activity of the culture remained high, the 84.9 and 88.1% on days 5 and 7, respectively. Chitosan salicylate at a concentration of 0.1% had a moderate fungistatic activity, inhibiting the mycelium growth of *C. sativus* by 30.5 and 42.5%. Note that the 0.1% chitosan salicylate in the diluted culture fluid of the producer strains insignificantly decreased the direct inhibitory effect on the mycelial growth of C. *sativus*, to 61.9 and 69.4% (Table 2).

1. Antagonistic activity of *Bacillus subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D in submerged culture added with chitin and chitosan (M±SEM)

Treatment	Zone free from test culture growth, mm	
	for *Alternaria solani*	for *Clavibacter michiganensis* subsp. *michiganensis* 101
Vitaplan, KZh (control)	40.3±1.0	31.9±1.0
Vitaplan, KZh + dry chitin (1.0 %)	37.8±0.5	31.9±1.2
Vitaplan, KZh + colloidal chitin (1.0 %)	43.6±0.8	34.8±1.8
Vitaplan, KZh + colloidal chitosan (1.0 %)	36.0±1.0	31.6±0.5
LSD_{05}	1.1	0.9

N o t e. For all treatments, the titers of the producer strains were 10^{10} CFU/ml.

2. Growth of the test culture of plant pathogen *Cochliobolus sativus* as influenced by various formulations of Vitaplan, KZh (M±SEM)

Treatment	Concentration of additional component, %	Viable cells, CFU/ml	Day 5		Day 7	
			diameter of colony, mm	growth inhibition, %	diameter of colony, mm	growth inhibition, %
Water (control)			52.5±0.5	–	80.0±1.2	–
Vitaplan, KZh		10^{10}	10.0±0.5	80.9	10.0±1.0	87.5
Vitaplan, KZh (diluted 1:10)		10^9	13.5±0.5	74.3	15.0±1.2	81.2
Vitaplan, KZh + colloidal chitin	1,0	10^{10}	8.0±0.2	84.9	9.5±1.0	88.1
Витаплан, КЖ (diluted 1:10) + chitosan salicylate	0,1	10^9	20.0±1.5	61.9	24.5±1.5	69.4
Chitosan salicylate	0,1		36.5±2.2	30.5	46.0±2.0	42.5
LSD_{05}				1.3		0.6

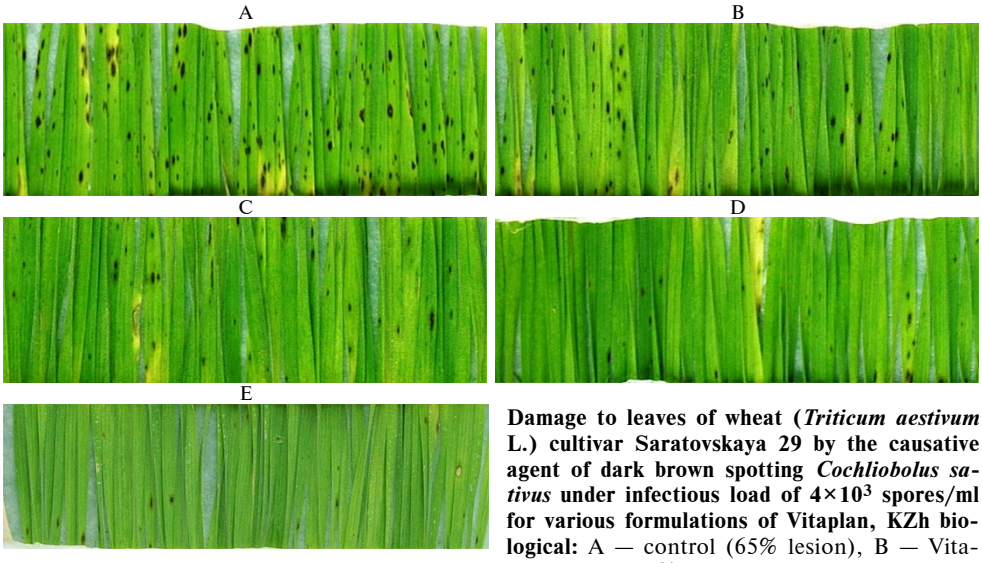
N o t e. Dishes means the absence of growth inhibition.

Upon infecting the leaves of susceptible wheat cultivar Saratovskaya 29 with a suspension of *C. sativus* spores in the control, the signs of the disease appeared as brown spots occupying 65 and 100% of the leaf area under the infectious load 4×10^3 and 20×10^3 spores/ml, respectively (Fig.). Preliminary spraying of plants with Vitaplan, KZh a dilution of 1:10 followed by infection with the causative agent of dark brown spotting reduced the area of leaf damage to 50 and 80%, depending on the infectious load of the pathogen.

A 1.0% concentration of colloidal chitin at in the growth medium for the strains producers reduced leaf damage to 40 and 50% at an infectious load of 4×10^3 and 20×103 spores/ml, which indicates a higher immunomodulatory activity of this formulation compared to Vitaplan, KZh at a dilution of 1:10. Chitosan salicylate showed itself to be an effective inducer of disease resistance, reducing plant damage to 10 and 20%. The 0.1% chitosan salicylate supplementation to Vitaplan, KZh diluted 10-fold also had a positive effect on the the antagonist, increasing its biological activity 2.0-2.5-fold with the leaf damage of 20 and 40% at an infectious load of 4×10^3 and 20×10^3 spores/ml (see Fig.).

Our experiments showed that the effect of Vitaplan formulations against dark brown spot depends on the intensity of damage to wheat plants by the causative agent of the disease *C. sativus*. Vitaplan, KZh at a dilution of 1:10 in

combination with 0.1% chitosan salicylate had the greatest protective effect (see Fig.).



Damage to leaves of wheat (*Triticum aestivum* L.) cultivar Saratovskaya 29 by the causative agent of dark brown spotting *Cochliobolus sativus* under infectious load of 4×10^3 spores/ml for various formulations of Vitaplan, KZh biological: A — control (65% lesion), B — Vitaplan, KZh (50%), C — Vitaplan, KZh + colloidal chitin (40%), D — Vitaplan, KZh + chitosan salicylate (20%), E — chitosan salicylate (10%).

Two mechanisms can underlay the efficiency of a biological product in protecting wheat from dark brown spotting, namely a direct suppression of the causative agent due to the synthesis of antibiotics and enzymes by producer strains and an indirect influence through the induction of systemic resistance. In our opinion, there are significant differences in the mechanisms of wheat protection against dark brown spotting for Vitaplan, KZh and its formulations containing colloidal chitin and 0.1% chitosan salicylate.

Vitaplan, KZh based on *B. subtilis* VKM B-2604D and *B. subtilis* VKM B-2605D strains exhibits suppressive effect due to high fungistatic activity against *C. sativus*, caused by the synthesis of a multicomponent metabolite complex which includes peptide and polyene antibiotics, while the inducing activity of the microbial preparation is, on the contrary, low (see Table 2). Depending on the infectious load, the Vitaplan, KZh formulations supplemented with colloidal chitin and chitosan salicylate reduced the disease sings 1.5-3.0-fold compared to the control. The main protective mechanism providing high biological activity of a formulation combining Vitaplan, KZh (1:10) and chitosan salicylate (0.1%) is induced resistance. Fungistatic activity of this composition was somewhat lower than that of Vitaplan, KZh (1:10). The biological activity of the form combining Vitaplan, KZh (1:10) with colloidal chitin (1.0%), apparently, was due to the combination of the fungicidal activity of the preparation and the induction of plant defense reactions. Thus, the formulations of Vitaplan, KZh that we have developed provided direct suppression of the pathogen and exerted an indirect protective effect through an increase in the disease resistance of wheat plants to dark brown spot.

According to contemporary concepts, microbe-plant interaction in the plant—plant pathogen—plant pathogen antagonist system are complex and multidirectional. For example, *B. subtilis* 26D induced systemic resistance in wheat plants upon infection with *Septoria nodorum* and in potato plants infected with *Phytophthora infestans* oomycete through the accumulation of H_2O_2 and an increase in the transcriptional activity of SC-regulated PR protein genes, in peroxidase activity, and lignin deposition at the sites of infection [40].

Elicitors triggering plant defense mechanisms can be proteins, lipopeptides, polysaccharides, and other compounds associated with the cell wall of *B. subtilis* [41]. Bacterial metabolites possessing the properties of induced resistance include a chain of interrelated defense reactions, e.g., formation of reactive oxygen species, phosphorylation of proteins, and the triggering of the basic mechanisms of plant immunity, which lead to the development of systemic resistance [42-45].

Chitin and chitosan are molecular determinants of many plant pathogenic microorganisms that are recognized by plant protein receptors [46]. Interactions with receptors activate a complex of defense reactions of nonspecific immunity to form a systemic resistance to pathogens. Induced protective reactions include i) generation of reactive oxygen species (ROS), ii) synthesis of callose, iii) strengthening of the cell wall with lignin, iv) development of a hypersensitivity reaction (HS reaction) which causes the death of plant cells and the pathogen in the zone of its introduction, v) induction of genes involved in synthesis of protective proteins, vi) synthesis of 18 classes of pathogen-induced proteins with antimicrobial (thionines, defensins, proteinase inhibitors) and lytic activity (chitinases, glucanases), vii) induction of defense hormones (abscisic acid, jasmonates, salicylic acid), and viii) induction of the phenol-propanoid pathway and an increase in the level of phytoalexins [47].

It is known that a mechanism associated with an increase in the tobacco plant resistance is an increase for SA under the influence of treatment with *B. pumilus* SE34 strain [48]. Since SA is a signaling molecule that triggers a cascade of defense reactions in plants, the exogenous SA together with *Pseudomonas fluorescence* (pf4-92) enhanced the inducing activity of the antagonist in protecting chickpea seedlings from fusarium wilt [49]. The combined use of *P. fluorescence* (SE21 and RD41) and resistance inductors (chitin and salicylic acid) stimulated plant growth and increased the efficiency of biological control of pepper rhizoctoniosis [50]. Several works confirmed that the combination of microbial antagonists and chitosan increase the effectiveness of biologicals in protecting vegetables and strawberries from powdery mildew [51]. Supplementation of the *Bacillus* sp. culture medium with chitin significantly reduced wilting of the cotton [52].

It is obvious that protecting plants from diseases requires new multifunctional microbiological compositions effective against a wide range of plant pathogens. Our previous studies have shown that the combined use of microbial strains and chitosan complexes is effective for protecting wheat from a complex of major diseases, including root rot and leaf spots, as well as for increasing yields [53].

So, we have developed two new formulations, the Vitaplan, KZh + colloidal chitin and Vitaplan, KZh + chitosan salicylate with increased biocidal activity and inducing properties compared to the original formulation Vitaplan, KZh. The Vitaplan, KZh + colloidal chitin formulation exerts high antagonistic activity. Colloidal chitin (1.0%) added to the medium for submerged culture of producer strains ensured both effective suppression of *Cochliobolus sativus*, the causative agent of wheat dark brown spotting (up to 84.9-88.1%), and a 1.5-2.0-fold increased inducing effect compared to the original biological product. Chitosan salicylate (0.1%) added to Vitaplan, KZh increases its biological activity 2.0-2.5-fold. High protective effect of the new optimized formulation of Vitaplan, KZh towards *C. sativus* that we revealed was apparently due to a combined action of metabolites produced by *B. subtilis* strains which suppress or detain growth of the plant pathogen and chitosan-based inducers of disease resistance. Our findings indicate that the combination of active strains of microbial antagonists of plant pathogens and chitosan complexes is promising for increasing the biological efficiency and expanding the spectrum of action of the developed formulations.

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BIOCONTROL OF AGRICULTURAL PESTS BASED ON AUTODISSEMINATION OF ENTOMOPATHOGENIC NEMATODES OF *Steinermatidae* FAMILY (*Nematoda: Rhabditida*)

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Abstract

At present, the development of biological plant protection systems is among the most important economic, social and environmental challenges. Creating an effective system is impossible without the combination of a variety of biological agents and pest control techniques. Combining a variety of pathogenic organisms and synthetic sex pheromones is a way to improve the situation. This paper shows the effectiveness of entomopathogenic nematodes (EPN) of the family *Steinernematidae* Filipjev, 1934 as an autodissemination agent for agro-ecosystems under crop rotation and at apple-tree orchard, and assessed the effect of introducing pathogens on indigenous entomopathogens in soil. In particular, a decrease in the number of harmful insects and an increase in the activity of natural beneficial entomopathogens have been demonstrated. The essence of the method consists in the targeted introduction of entomopathogenic bioagents into the agro-ecocenosis by means of their application to attracted insects caught in traps, and thus creating an epizootic in the populations of target species. Previously, entomopathogenic nematodes were not used as autodissemination agents against superdominant species, the codling moth *Cydia pomonella* L., 1758 and click beetles of the family *Elateridae* Leach, 1815; moreover, their effect on other members of the entomofauna of agro-ecocenoses has not been studied either. The purpose of this work was to evaluate the effectiveness of the EPN autodissemination method for various cultures. The successful testing of Granulosis virus dissemination method in the apple orchard and the EPN autodissemination against wireworms prompted us to conduct the investigation reported herein. Two species of entomopathogenic nematodes of *Steinernematidae* family, the *Steinernema carpocapsae* (Weiser, 1955) and *St. feltiae* (Filipiev, 1934) were reproduced in lab culture in different host insects to produce nematode inoculums. The experiments found out that specially designed formulations and modified pheromone traps ensure EPN introduction into the agrocenoses due to nematode invasion of trapped insects followed by their free flight to spread pathogens. As a result, the nematode-bacteria complex occurred in 60.0-100 % of click beetles of the *Elateridae* family and 34.0-35.3 % of *C. pomonella* L. and *Grapholitha molesta* (Busck, 1916). This indicates accumulation of biocontrol agents in the soil of the agrocenoses due to EPN introduction. The EPN autodissemination application also reduced the damage to apple fruits by up to 10 %, and corn and soybean plants by 13,2 % compared to areas where chemical treatments were applied. The method has no negative impact of EPN on green lacewings (*Chrysopidae* Schneider, 1851) and the *Hymenoptera* of the families *Braconidae*, Latreille, 1829 and *Ichneumonidae*, Latreille, 1802, the predators of insect pests. In the garden where the tests were carried out, there was a 15 % increase in infection of caterpillars of *C. pomonella* by *Hymenoptera*. It is established that the EPN autodissemination stimulates the activity of indigenous soil EPN, leading to a 1.5-2.0-fold increase in the number of trapped nematodes in the bioassay test compared to the period prior to EPN autodissemination. Importantly, the effect of autodissemination turned out to be prolonged and manifested the next year both in the apple orchard and in the crop rotation of

agricultural crops.

Keywords: biocontrol, entomopathogenic nematodes, autodissemination, soil nematodes, codling moth, click beetles, wireworms, pheromone traps, apple trees, maize, soybean

The excessive use of chemical pesticides in Russia raises concerns about the safety of products and the environment, therefore, natural regulators of biocenotic relations in agricultural systems is becoming a new strategy for protecting plants from harmful organisms [1].

Use of synthetic insect sex pheromones is a technology of pest population biocontrol on the most important agricultural crops [1-5]. These attractants used worldwide for monitoring, mass capture and disturbance of reproductive connections (disorientation) of phytophages have not yet exhausted themselves [6-11]. For many insect species in the agrocenosis, it was found that with an increase in the concentration of synthetic sex pheromone, egg laying decreases, time for development of preimaginal stages lengthens and their survival decreases, which, in particular, has been demonstrated in the garden for apple and eastern moth [4, 12 -15]. The search for new methods of using insect sex pheromones to control pest population led to the discovery of original methods based on the dissemination of pheromones and autodissemination of entomopathogens of target species using pheromone traps with applicating devices [15, 16].

Entomopathogenic nematodes (EPN) of the families *Steinernematidae* Filipjev, 1934 and *Heterorhabditidae* Poinar, 1976 are effective natural bioagents. These organisms can infect more than a thousand species of arthropods, many of which are dangerous pests of the most important agricultural crops [17-20). Among biological agents produced in the world, nematode preparations are in second place after bacterial ones [21-25]. EPNs have the ability to independently penetrate into the prey, survive in dead insects and contribute to the invasion of other pathogens (in particular, viruses and bacteria) of entomopathogenic parasites into the insect body. The high development rate of nematodes (in the body of a living host for 6-10 days) allows them to spread with larvae and imago of pests [26-31]). The only factor limiting the widespread use of EPN-based drugs is their high hygrophilicity. Therefore, to increase the viability of these bioagents, various formulations are developed that provide sufficient and prolonged moisture supply to nematodes [17, 23-26]. It is also important that these pathogens, interacting with other consorbents of the biocenosis, can also play a microregulatory role in the formation of the soil structure [32, 33]. The peculiarities of the EPN biology suggest the possibility of saturating the soils of agrocenoses with them using males caught with pheromone traps with applicators which infect attracted insects with entomopathogenic nematodes (the method of autodissemination of entomopathogens). Many studies have shown a high biological effectiveness of EPN against wireworms (larvae of click beetles of the family *Elateridae*) and the codling moth *Cydia pomonella* L., 1758, and various ways of their use were considered [23, 34-36].

Here, we propose a new method for application of entomopathogenic nematodes by autodissemination which ensures their long life span, high activity and speedy propagation. The method is based on intra- and interspecific chemical communication and positive phototaxis of insect species attracted by synthetic sex pheromones or light into the applicating devices [16]. Insects leaving the applicator act as EPN carriers used to protect agricultural crops from dominant pests [16, 35].

Our goal was to assess whether pheromone traps can be used for autodissemination of entomopathogenic nematodes as biocontrol agents for the fruit moth *Cydia pomonella* L., 1758 and click beetles from the family *Elateridae* Leach, 1815,

and how they affect the beneficial fauna in agrocenoses.

Materials and methods. Three species and four variants of entomopathogenic nematodes (*Steinernematidae* family), the *Steinernema feltiae* (Filipiev, 1934), *St. kraussei* Steiner, 1923, *St. carpocapsae* (Weiser, 1955), *St. carpocapsae* var. "*agriotes*" (Weiser, 1955) from the collection of useful organisms of the Federal Scientific Center for Biological Plant Protection (FSCBPP) were used. At first *St. kraussei* was derived from the collection of the All-Russian Research Institute of Fundamental and Applied Parasitology of Animals and Plants RAS (VNIIP, Moscow), *St. feltiae* and *St. carpocapsae* var. "*agriotes*" were obtained from the collection of the All-Russian Research Institute of Plant Protection (VIZR, St. Petersburg—Pushkin). *St. carpocapsae* is a local form found in an apple orchard in the Leningradskaya village (Krasnodar Territory).

The ESP was propagated in the laboratory insect hosts greater wax moth *Galleria melonella* (Linnaeus, 1758) and yellow mealworm beetle *Tenebrio molitor* Linnaeus, 1758 (an MLR 35 OH artificial climate chamber, Panasonic, Japan) in as per Danilov's method [24] with our modifications [35, 36].

The detection of entomopathogenic nematodes in soil samples from the biotopes was carried out according to Spiridonov [27], using 10 caterpillars of *G. melonella* placed in each soil sample taken at control sites and the sites of autodessemination. After 1 week, the caterpillars were removed and the entomopathogens were detected (a microscope Biolam, LOMO, Russia, 90× magnification); soil samples were also examined under a binocular microscope at 10× magnification (MBS-10, LZOS, Russia) [27].

After autodissemination, entomopathogens were detected in adults, larvae, and caterpillars using the so-called "nematode traps" and by viewing the biomaterial under a microscope (Biolam, LOMO, Russia, 90× magnification).

To infest three species of click beetles, the Kuban *Agriotes tauricus* Heyden, 1882, common click beetle *A. sputator* (Linnaeus, 1758), and steppe wireworm *A. gurgistanus* (Faldermann, 1835), modified standard Estron-type traps (manufactured by the All-Russian Plant Quarantine Center, Moscow Province) were used. A 45×95 mm foam rubber sponge impregnated with an EPN suspension with the titer of invasive larvae 2.5×10^6/ml was put inside. To ensure free migration of captured insects into the environment, a 35 mm flight hole was made in the insect receiver. Ten traps per each treatment were set depending on the beginning of flight period of each studied click beetle species, distributed evenly over the soybean and corn plots at a distance of 30-40 m from each other according to the "envelope" scheme. For proper isolation, the test sites were located at a 200 m distance. A freshly prepared suspension of nematodes was added to sponges and sampling was performed every 7-10 days during the entire flight period of insects. To count the captured male click beetles, half of the traps were without an air hole. The number of the infected beetles and invasive larvae of entomopathogenic nematodes released from them were determined in lab tests.

For dissemination of EPN in the apple orchard, we used standard modified traps of the Atracon-A type (made by us from Tetrapak paper), 10 traps per treatment. To apply nematodes, a 20×20 mm foam rubber sponge with a suspension of entonematodes (a titer of invasive larvae of 2.5×10^6/ml) was placed inside. To determine the number of caterpillars infected with nematodes, we used traps with glue inserts; for the further spread of EPN in the agrocenosis, half of the traps did not contain glue inserts.

Every 7-10 day, a freshly prepared suspension of nematodes was applied

to the sponges and sampling was carried out (the frequency of changing the biological product we have previously determined to ensure its effectiveness with clickers) [35]. The insects caught using traps and trapping belts trapped were counted, and the degree of infestation of adults and larvae by entomopathogens was determined in lab tests.

Field trials with the codling moth was carried out in the apple orchards of the Kuban Uchkhoz (Trubilin Kuban State Agrarian University). Infected insects were caught on a 1-hectare area. The number of captured infected insects were compared to that of the control (pesticide-treated) plots located at a distance of at least 500 m from the test plots.

Entomophages were isolated from the codling moth caterpillars caught with hunting belts followed by individual hatching.

The collected biomaterial was identified using the fundamental keys of the Zoological Institute RAS (St. Petersburg) and Far East Branch RAS (Vladivostok) [37, 38]. MLR 35 OH climate chambers were used to keep caterpillars and pupae of the codling moth to ensure either the emergence of entomophages from infected insects, or the emergence of butterflies. Microscopy was performed using MBS-10 binocular microscope (LZOS, Russia, 8× magnification).

Experimental data were statistically processed according to Dospekhov [39] using the Statistica 12.6 program (StatSoft, Inc., USA). The tables and figures show the means (*M*) and standard errors of the mean (±SEM). The significance of differences between the options was determined using the Student's *t*-test at $P \geq 0.95$.

Results. Lab screening the FNCBZR collection of entomopathogenic nematodes from various biotopes of the Krasnodar Territory reveled that three species (*St. carpocapsae*, *St. feltiae*, and *St. kraussei*) had the highest activity towards *G. pomonella* caterpillars and two species (*St. carpocapsae* var. "*agriotes*" and *St. feltiae*) towards wireworms [35, 36]. These species were involved in further studies.

Entomopathogenic nematodes are quite widespread in some biocenoses, and their main habitat is soil. Thereof, before studying the effect of introduced pathogens on the aboriginal pathogens of insects, we examined soils from biotopes in the experimental sites for the presence of entomopathogenic nematodes. In the apple orchard of FNCBZR intensively exploited for several years, we found *St. carpocapsae* and *Steinernema* sp. of the family *Steinernematidae*, hence, for dissemination we used *St. feltiae* nor found in the ecosystem that was chosen. According to our observations and data obtained earlier [40], a low number or almost complete absence of these pathogens are characteristic of row crops in crop rotation. A similar situation was seen in 2013 in the garden of the Educational farm Kuban, which was not used for 2 years before the research. I.e., the number of *St. carpocapsae* and *Steinernema* sp., caught with a bait insect, was lower here than in the FNCBZR orchard.

The test autodissemination of EPN against click beetles showed that the Kuban click beetle poses the greatest danger to the seedlings of soybeans and maize. In pheromone traps the number of beetles caught on maize and soybeans was 405.0±3.5 and 231.3±5.7, respectively, in 2011, 275.3±8.3 and 109.4±7.6 in 2012, and 119.7±7.6 and 86.7±7.6 in 2013 (Fig. 1). The abundance of common click beetle *A. sputator* was lower, 101.3±2.4 and 65.7±6.2 on maize and soybean in 2011, 76.6±6.0 and 38.7±3.6 in 2012, 42.0±5.6 and 32.0±3.6 in 2013 (see Fig. 1). The number of caught males of the steppe wireworm *A. gurgistanus* was minimum, 7.6±2.5 and 5.0±1.7 on maize and soybeans, respectively, in 2011, 6.3±1.5 and 3.7±0.6 in 2012, and decreased to 2.6±1.5 and 1.3±0.5 in 2013 (see Fig. 1).

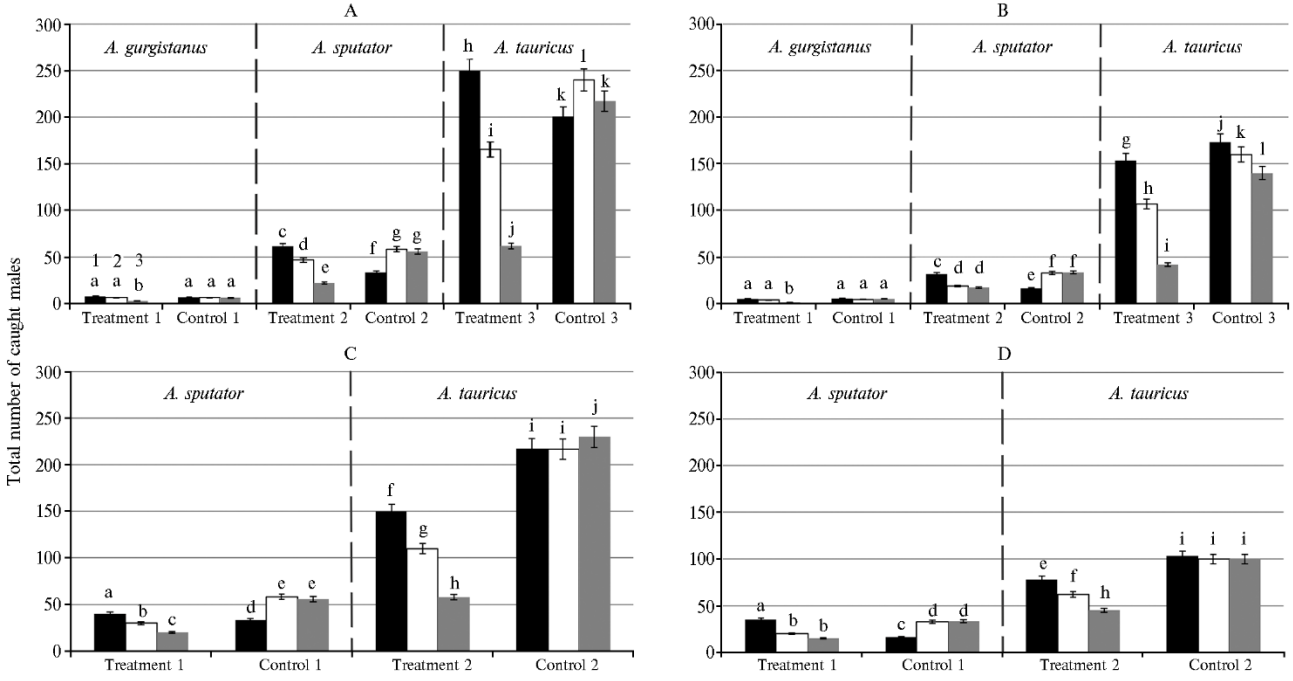

Fig. 1. Number of *Agriotes* click beetles in crops due to autodissemination: A and B — nematode *Steinernema carpocapsae* (maize and soybeans, respectively), C and D — nematode *St. feltiae* (maize and soybeans, respectively); I-III — application of nematodes and controls without application; 1, 2, 3 — 2011, 2012, and 2013 ($n = 10$, $M \pm$SEM, crop rotation, FNCBZR, Krasnodar Territory). Different letters mark statistically significant differences at $P \geq 0.95$. For the experimental design, see the *Materials and methods* section.

1. Efficiency of entomopathogenic nematodes *Steinernema carpocapsae* and *St. feltiae* towards *Agriotes* click beetles upon autodissemination in crops of maize and soybeans (n = 10, M±SEM, crop rotation, FNCBZR, Krasnodar Territory)

Click beetles	Nematodes	Number of caught males								
		2011			2012			2013		
		total	infested		total	infested		total	infested	
			total	%		total	%		total	%
		Maize								
A. sputator	*St. carpocapsae*	73.0±7.0	65.0±5.6	89.0	54.0±5.6	54.0±5.6	90.0	55.0±4.6[a]	47.0±6.0	85,4
Conrol		63,0±4,6	0	0	75.0±6.0	0	0	87.0±5.0	0	0
A. sputator	*St. feltiae*	40.0±5.0	32.0±3.5	80.0	26.0±1.8	26.0±1.8	86.7	23.0±2.6[c]	20.0±3.3	86,9
Conrol		40,0±4,0	0	0	50.0±5.5	0	0	60.0±6.2	0	0
A. tauricus	*St. carpocapsae*	150.0±10.3	147.0±7.8	98.0	88.0±2.7	88.0±2.7	97.7	60.0±7.2[e]	57.0±5.2	95,0
Conrol		150,0±8,0	0	0	160.0±9.3	0	0	160.0±9.6	0	0
A. tauricus	*St. feltiae*	68.0±4.6	61.0±6.2	89.7	33.0±3.6	33.0±3.6	94.2	25.0±4.3[g]	23.0±4.3	92,0
Conrol		70,0±4,3	0	0	79.0±6.2	0	0	85.0±6.5	0	0
A. gurgistanus	*St. carpocapsae*	7.0±1.8	6.0±3.6	85.7	4.0±1.0	4.0±1.0	80.0	4.0±2.5[i]	3.0±1.2	75,0
Conrol		5,0±2,5	0	0	6.3±1.5	0	0	7.0±1.0	0	0
		Soybeans								
A. sputator	*St. carpocapsae*	30.0±6.1	27.0±4.3	90.0	23.0±3.6	23.0±3.6	92.0	15.0±2.6	15.0±2.6	83,3
Conrol		31,0±4,8	0	0	38.0±3.6	0	0	43.0±6.9	0	0
A. sputator	*St. feltiae*	17.0±3.6	15.0±2.8	88.2	8.0±1.6	8.0±1.6	80.0	7.0±1.6	7.0±1.6	87,5
Conrol		15,0±4,2	0	0	21.0±2.6	0	0	25.0±3.6	0	0
A. tauricus	*St. carpocapsae*	130.0±6.2	122.0±6.9	93.8	63.0±7.9	63.0±7.9	90	47.0±5.3	47.0±5.3	79,6
Conrol		130,0±7,9	0	0	146.0±8.5	0	0	150.0±6.6	0	0
A. tauricus	*St. feltiae*	50.0±4.9	45.0±5.3	90.0	26.0±4.0	26.0±4.0	92.8	21.0±2.1	21.0±2.1	95,4
Conrol		48.0±5.2	0	0	53.0±6.3	0	0	60.0±6.5	0	0
A. gurgistanus	*St. carpocapsae*	5.0±1.5	3.0±0.8	60.0	2.0±0.4	2.0±0.4	66.7	2.0±0.2	2.0±0.2	100
Conrol		4.0±1.3	0	0	6.0±1.8	0	0	5.0±1.3	0	0

Note. Controls — without application of nematode autodissemination method. Different letters indicate statistically significant differences in the number of caught insects between the tests and controls in different years and on different crops at $P \geq 0.95$.

Thus, when using the autodissemination method for 3 years, the number of insects caught in pheromone traps decreased while in the control, it either did not change, as for the steppe wireworm (in 2011-2013, 6.0±1.0-6.6±1.5 and 4.3±1.1-5.3±0.6 individuals caught on corn and soybeans), or increased, as for the Kuban click beetle. The Kuban click beetle increased in abundance from 430.0±8.3 in 2011 up to 457.3±6.4 in 2013 on maize, the number of common click beetle increased from 66.6±3.7 in 2011 to 111.4±3.9 specimens in 2013 om maize. On soybeans, the male common click beetle also increased in number from 32.3±1.2 in 2011 up to 66.6±5.7 in 2013 (see Fig. 1). There was also a slight decrease in the number of captured males of the Kuban click beetle in the control on soybeans (from 276.6±5.7 in 2011 to 240.0±9.6 in 2013), however, the differences with the test treatments remained statistically significant at $P \geq 0.95$ (see Fig. 1).

The imagoes died in 4-5 days, which created conditions for the spread of infection. The number of individuals infected with *St. carpocapsae* was up to 83.3-92.0% for *A. sputator*, 79.6-98.0% for *A. tauricus*, and 60.0-100% for *A. gurgistanus*, Infestation by *St. feltiae* occurred in 80.0-88.2% of *A. sputator* and 9.7-94.2% of *A. tauricus* (Table 1). The number of released invasive nematode larvae per insect was 8.8×10^4 for *A. sputator*, 9.1×10^4 for *A. gurgistanus*, and 1.25×10^5 for *A. tauricus*, which suggests the introduction of more than 10 million entomopathogens into the environment and EPN activation in natural populations.

Additional introduction of nematodes into the soil, according to our findings and as previously noted by Danilov et al. [41], can cause a change in insect ethology. In wireworms, larvae infected with nematodes crawl out to the soil surface, becoming, as a result, more accessible to entomophages (carnivorous ground beetles) and vertebrates.

Note, in 2011-2013, there was a decrease both in the number of male click beetles caught in pheromone traps and damage to maize and soybean plants by pests by 13.2% compared to the use of chemical insecticide Cruiser®, KS (Syngenta, Switzerland) for seed treatment. Larvae of click beetles were also not found in the soil excavations. These were the result of the dissemination of entomopathogenic nematodes in 2011-2013.

2. Efficiency of entomopathogenic nematodes *Steinernema carpocapsae* and *St. feltiae* upon autodissemination in apple orchard (n = 10, M±SEM, Krasnodar Territory, 2013-2015)

Treatment	Number of caught insects							
	total				infested by nematodes, %			
	phytophages		entomophages		phytophages		entomophages	
	Cydia pomonella	*Grapholitha molesta*	*Chrysoperla carnea*	*Hymenoptera*	*Cydia pomonella*	*Grapholitha molesta*	*Chrysoperla carnea*	*Hymenoptera*
Kuban orchard								
Test	20.0±1.7[a]	0	0	20.0±0.6[c]	30.3	0	0	0
Control	40.0±2.2[b]	0	0	10.0±0.8[d]	0	0	0	0
FNCBZR orchard								
Test	37.0±3.5[a]	99.0±3.5[e]	9.0±1.1[g]	62.0±1.9[i]	35.3	34.0	0	0
Control	45.0±3.3[b]	120.0±4.1[f]	2.0±0.6[h]	10.0±1.7[j]	0	0	0	0

Note. Controls — without application of nematode autodissemination method. Different letters indicate statistically significant differences in the number of caught insects between the tests and controls at $P \geq 0.95$.

EPN were also autodisseminated in apple orchards for 3 years in two plots with different levels of pre-application of chemicals. Our studies have shown the possibility of using nematodes for autodissemination against the codling moth, since codling moth butterflies *Cydia pomonella* infected with pathogens were found in the traps during monitoring of pest abundance in both orchard agrocenoses, and the eastern codling moth *Grapholitha molesta* (Busck, 1916) also in

the FNCBZR orchard (Table 2). The identified percentage of *Lepidoptera* infested by entomopathogens was approximately the same, 30.3-35.3%. The number of helminths released from one insect was 1×10^4 for *C. pomonella* and 1×10^3 for *G. molesta*.

In the orchards, as in the crop rotation, we revealed a decrease in the number of insects caught in traps. There was a decrease in fruit damage (by about 10%) compared to standard protection systems, given that even in ecological gardens at least 4-5 treatments with various chemicals are carried out [6].

Among the captured entomophages, the *Chrysoperla carnea* St. and *Hymenoptera* (Linnaeus, 1758), namely, *Ascogaster quadridentatus* Wesmael, 1835, *A. rufidens* Wesmael, 1835, *Microdus rufipas* Nees, 1814 of the family *Braconidae* Latreille, 1829, and *Liotryphon crassisetus* (Thomson, 1877), *L. caudatus* (Ratzeburg, 1848), *L. punctulatus* (Ratzeburg, 1848) of the family *Ichneumonidae* Latreille, 1802, we did not identify insects infected with nematodes.

In other words, certain groups of entomophages turned out to be tolerant to the effects of entomonematodes. Back in 2008, Danilov et al. [41] hypothesized that the constant use of EPN in an apple orchard for a number of years contributes to an increase in both the quantitative and qualitative diversity of the species composition of entomophages. Our study has confirmed this hypothesis. The number of representatives of *Hymenoptera* identified in the second half of August was significantly higher. In the FNCBZR orchard where the method was tested we recorded an increase in the species diversity of *Hymenoptera* (from four species to six species) and the infection rate of *C. pomonella* caterpillars from 6% to 15% (Fig. 2).

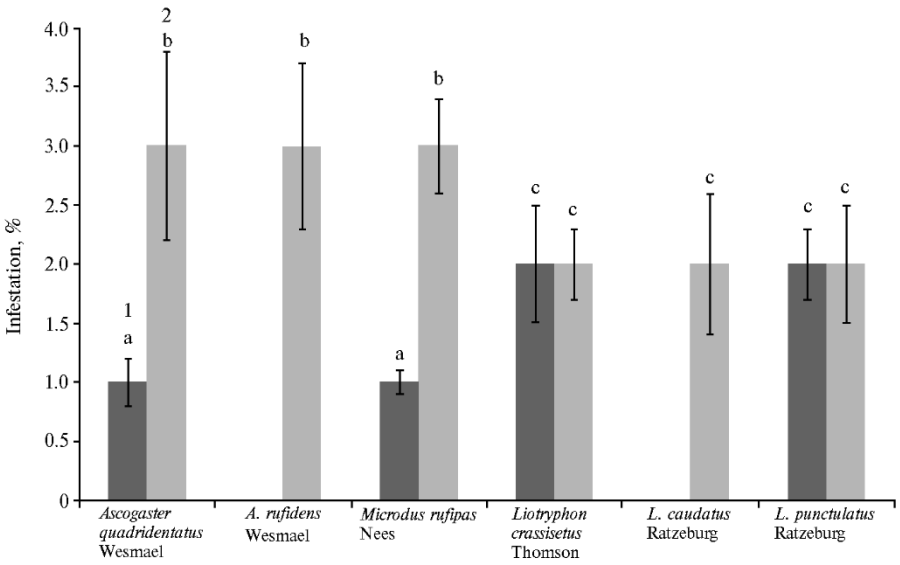

Fig. 2. Infestation of *Cydia pomonella* by entomophages of the families *Braconidae* and *Ichneumonidae* (*Hymenoptera*) in 2013 (1) **and 2015** (1) (100 insects in total, four repetitions; *M*±SEM, the FNCBZR orchard, Krasnodar Territory). Different letters indicate statistically significant differences in the number of entomophages between the years and $P \geq 0.95$.

A number of works [16, 42, 43] report that entomopathogenic fungi and viruses are agents mainly and quite successfully used for autodissemination in traps of various types (feromon, light, etc.). The entomopathogenic nematodes are traditionally applied by spraying, irrigation, treatment of the soil prior to crop sowing and near-tree rings in orchards, etc. [21, 44, 45]. The method of biological control of pests by means of EPN autodissemination that we propose in this paper is

another technique to use these entomopathogens.

Our research has demonstrated beneficial effect of the autodissemination of EPNs on increasing the invasive activity of natural populations of entomohelminths in the apple orchard. Soil biotests with *G. melonella* showed an increase in the number of invasive EPN larvae of the species *St. carpocapsae* per caterpillar two months after autodissemination compared to that prior to our experiment (Table 3). Danilov et al. [41] reported about an increased activity of local populations of pathogens upon the introduction of new species into the apple orchard agrocenosis, but these authors applied biologicals based on suspensions of entomopathogenic nematodes to near-tree rings. There are data, for example, reported by Somasekhar et al. [46], on the positive effect of introduced steinermatids on aboriginal nematode species of soils in agrocenoses.

In experiments, we also detectedhe nematode *St. carpocapsae* in the soil under the grain-row crop rotation, and pathogens persisted not only during the entire period of the study. As a result, stable foci of infection emerged, acting for several years (see Table 3).

Comparison of the number of *St. carpocapsae* caught in test to that under traditional protection from pests showed positive dynamics, while this did not occur in the control (see Table 3).

3. Number of *Steinernema carpocapsae* larvae caught in soil (bio-test with *Galleria melonella*) **upon autodissemination of nematodes of the *Steinernematidae* family** (*M*±SEM, Krasnodar Territory, 2011-2015)

Conditions	Depth, cm	
	5	10
Crop rotation of FNCBZR (2011-2013)		
Prior to the experiment	0	0
After the experiment:		
in 2 months	50.0±4.2	20.0±2.3
in 1 year	40.0±2.1	20.0±1.5
Control (without autodissemination)	0	0
Kuban orchard (2013-2014)		
Prior to the experiment	20.0±2.6	0
After the experiment:		
in 2 months	50.0±3.1	30.0±1.8
Control (without autodissemination)	17.0±2.2	0
FNCBZR orchard (2014-2015)		
Prior to the experiment	90.0±3.3	50.0±2.9
After the experiment:		
in 2 months	150.0±4.0	100.0±3.9
in 1 year	140.0±4.6	110.0±4.1
Control (without autodissemination)	80.0±3.3	40.0±1.9

Thus, we have found out that autodissemination of entomopathogenic nematodes of the *Steinernematidae* family are suitable to protect maize, soybeans and apple orchards from a various pests. Entomopathogenic nematodes disseminated by autodissemination infected 60.0-100% of male click beetles of the *Elateridae* family and 30.3-5.5% of apple and eastern moth butterflies, that is, the proposed method stands along with traditional methods of introducing pathogens in agroecosystems, especially in an organic garden where all chemicals are prohibited. Importantly, both in an apple orchard and crop rotations, the autodissemination methods affect certain groups of insects without causing harm to beneficial organisms. In addition, in all areas where autodissemination tests were carried out, the introduction of a species of pathogen into the agrocenosis favorably influenced on the invasive activity of local populations of entomopathogenic nematodes.

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SCREENING FOR PROMISING YEAST STRAINS FOR SHERRY WINE PRODUCTION USING GENETIC AND ENOLOGICAL MARKERS

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Abstract

Flor yeast is a special group of wine-making microflora used in the production of biologically aged wines. In the process of biological aging, flor yeast, cultivated on the surface of dry, alcohol-based wine materials, switches its metabolism from enzymatic to oxidative, which leads to deep biochemical changes in the wine with the formation of unique features of the bouquet and aroma of sherry wines. Genetic, biochemical and physiological features of flor yeast associated with their adaptation to specific conditions of sherry winemaking have been studied in sufficient detail. Earlier, the use of comparative genomic analysis methods allowed us to identify a number of genetic markers specific for flor strains, convenient for searching for strains that are promising for producing sherry-type wines. This work presents the results of using a combined approach based on primary molecular genetic screening followed by analysis of physiological, biochemical and oenological properties for the selection of strains promising for sherry winemaking in a sample of 47 strains from the Magarach Winemaking Microorganism Collection and 96 natural isolates from samples of grapes in different climatic zones in the Republic of Crimea and in the Rostov region. At the first stage, the selection of promising strains was carried out on the basis of the results of genotyping by the presence of alleles of the loci ITS, *YDR379C-A*, and *FLO11* characteristic of flor strains. According to the genotyping data, the flor allele of the ITS locus was identified in 41 strains, the flor allele of the *YDR379C-A* gene was identified in 41 strains, and a deletion of 111 nt in the promoter of the adhesin *FLO11* gene, typical of flor strains, was found in only 12 strains. A total of 59 studied strains had the flor allele of at least one of the three loci. Further study of oenological properties showed that the presence of the ITS flor locus can serve as a marker for the selection of strains with high oxidative capacity. Film formation in most strains (11 samples out of 12) with a flor variant of the *FLO11* gene promoter confirmed the main role of this adhesin gene in determining the ability of strains to surface growth on wine material. The most informative was the use of the *YDR379C-A* marker. Of the 12 strains for which the presence of only this flor locus was determined, three collection and two natural strains (I-133, I-492, I-616, No. 49, No. 78) showed the presence of all flor characteristics. The formation of a continuous film with a reproducibility of 100 % was observed in two collection strains from this group — I-133 and I-492. Nine strains (75 %) synthesized aldehydes in an amount exceeding 100 mg/l, 6 strains (50 %) formed a film with varying degrees of formation from islands on the surface to continuous growth and film reproducibility from 33 to 100 %. Aldehyde aroma and oxidation taste were determined for 58 % of the samples. Analysis of the oenological properties of six strains with three flor loci showed that all of them formed a continuous film on the surface of the fermented wort at optimal times, and five strains synthesized aldehydes during alcoholic fermentation above 100 mg/l. Tasting laboratory samples of fermented wort revealed the presence of sherry tones in aroma and taste. According to the results of genotyping and oenological sherry characteristics, these strains were close to the control strain I-329. As a result, according to the results of the screening and production check, the

collection strain I-271 can be recommended for sherrying wine materials from the Aligote grape variety, traditionally used for these purposes, as well as blended wine materials from the Rkatsiteli and Sauvignon green grape varieties, new for sherry wines, which opens up the prospect creation of new brands of this type of wine. Thus, the study showed that during the initial selection of new flor strains of S. *cerevisiae* yeast, testing them for genetic markers ITS, *YDR379C-A*, and *FLO11* allows you to quickly and reliably identify the most promising strains for the technology of sherry wine production. Of 143 strains of wine yeast, according to the results of genotyping, taking into account oenology, strain I-271 with great potential for sherrying wine materials was identified.

Keywords: *Saccharomyces cerevisiae*, flor yeast, DNA markers, flor alleles, ITS, *YDR379C-A*, *FLO11*, winemaking, oenological properties, film formation, aldehyde synthesis, adhesins

Breeding industrial flor yeast strains specific to regional raw materials and winemaking technologies is a tool to intensify wine industry and produce a certain type of wine.

Advances in genetics, chemistry and biochemistry provide in-deep study of microbiology of winemaking to develop a new methodology for selecting flor yeasts. This significantly improves genetic fund of yeast strains for winemaking and gives fundamental and practical knowledge about their properties.

This concept is most relevant for yeast generating Jerez wines. The *Saccharomyces cerevisiae* [1-3] can form a biofilm (flor) on the wine surface and, due to oxidative metabolism, generate a characteristic sherry tone of the wine. Currently, studies of *S. cerevisiae* strains having wide practical use are actively developing [4-6]. For Jerez type wine, molecular methods ensure genetic identification of *S. cerevisiae* sherry yeasts to select biotechnologically effective strains and to ensure purity of their populations during wine production.

In early reports, the analysis of mtDNA polymorphism [7], microsatellite analysis [8], comparative genomic hybridization [9], and proportion of polymorphic loci quantified genetic diversity and phylogeny of sherry strains. Thus, the RFLP (restriction fragment length polymorphism) analysis of the ITS1 locus distinguished wine, Spanish, and French sherry yeasts [8, 10]. Most Spanish strains carry a 111 nt deletion in the *FLO11* gene promoter, leading to an increase in this adhesin expression and a denser film formation [11].

The rapid progress in genomic research has opened up new opportunities in disclosing structure, functioning, and evolution of the sherry yeast genome. In our studies and in the works of other researchers, the methods of comparative genomics revealed numerous genes specific for sherry yeast and alleles of genes that control various pathways of metabolism, transport, cell wall biogenesis, and stress resistance [12, 13], putatively associated with the adaptation of sherry strains to the specific conditions of biologically aged wines. Comparative genomics is important for understanding the origin and evolution of sherry strains [14-16] and allows us to develop convenient genetic markers for targeted selection of new sherry strains of *S. cerevisiae* yeast (17, 18). Genome-wide analysis of wine and sherry yeasts revealed a difference in the *YDR379C-A* gene sequence [17, 18].

Until recently, Russian winemakers used multiple alternations of mutagenesis and selection to create promising strains of wine yeast. This work, for the first time shows that testing of *S. cerevisiae* isolates for genetic markers ITS, *YDR379C-A* and *FLO11* allows rapid and reliable identification of yeast strains for the sherry vinification technology.

This research aimed at a multistage selection of new *Saccharomyces cerevisiae* yeast strains for the biofilm-based production of the Jerez-type wines using genetic markers in combination with oenological characteristics.

Materials and methods. A set of 143 *Saccharomyces* strains comprised 96 field isolates from grape sampled in different climatic zones (the Republic of Crimea and the Rostov region) which were genotyped and oenologically described in

part by us earlier [17] and 47 strains from the Magarach Winemaking Microorganism Collection (KMV Magarach) not recommended for the production of Jerez-type wine. The collection strain I-329 for the production of Jerez-type wine was a reference [19]. The media and cultivation modes matched the requirements and recommendations for the of Jerez wines [20, 21].

The genotyping for ITS, *FLO11*, and *YDR379C-Ac* markers was performed as described [17].

For DNA preparations, washed and lyophilized yeast cells (30-50 mg) were incubated with 500 μl of lysis buffer containing SDS (0.1 %), Triton X-100 (1%), and proteinase K (100 U/ml, Merck, Germany) for 20 min at 65 °C followed by phenol:chloroform (1:1) extraction and ethanol precipitated. The precipitate was dissolved in TE buffer (pH 8.0).

The Restriction Fragment Length Polymorphism (RFLP) analysis of PCR fragments of rDNA repeats, including two internal transcribed spacers ITS1 and ITS2 and the 5.8S rRNA gene, were used to identify and attribute the samples to wine or sherry yeast strains [8]. For PCR, primers Its1 5′-TCCGTAGGTGAACCTGCGG-3′ and Its4 5′-TCCTCCGCTTATTGATATGC-3′ were used [8]. The PCR mode was as follows: 96 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s (30 cycles). Hereinafter, a Mastercycler personal amplifier (Eppendorf, Germany) and a GoTaq® Flexi DNA polymerase were used as per the manufacturer's protocol (Promega, USA). The resulting fragments were treated with restriction endonuclease HaeIII (NEB, UK), and after analysis of electrophoregrams (1.5% agarose LE2, Lonza, Switzerland; molecular weight marker GeneRuler 1 kb DNA Ladder, Thermo Fisher Scientific, USA) the isolates were attributed to wine or sherry strains; the 311, 230, 172, and 128 bp fragments were characteristic for the *S. cerevisiae* wine strains while 311, 230, 148, and 129 bp fragments for the sherry strains.

To analyze the *FLO11* gene promoter polymorphism, strains with full-length and truncated (having a 111-nt deletion) *FLO11* gene promoter were identified by PCR technique using primers Flo11D.REV (5′-TTGGGCGACATTTTTCTTGT-3′) and Flo11D.FOR (5′-CCACGGGTGAGATTTGTTCT-3′) [11]. The PCR mode was 96 °C for 40 s, 51 °C for 40 s, 72 °C for 40 s (30 cycles). The fragments of approx. 400 nt or 300 nt in size characterized the wild and Jerez alleles of the *FLO11* gene, respectively [11].

To detect strains with polymorphic *YDR379C-A* gene, RFLP analysis with PCR primers F_sdh6 (5′-TCGCGTCAACTTGTTTTTGAG-3′) and R_sdh6 (5′-ATTCGTCAGTTCAG-3′) was applied. The PCR mode was 96 °C for 40 s, 52 °C for 40 s, 72 °C for 40 s (30 cycles). Fragments approx. 800 bp in length were treated with restriction enzyme AflIII (NEB, Great Britain) as per the manufacturer's protocol [18]. Restricts of 450 and 350 bp were characteristic of the sherry alleles of the *YDR379C-A* gene.

In lab tests, strains were cultured on one batch of must from Aligote grapes harvested in 2018 (the mass concentration of sugars 210 g/l, of titratable acids 7.0 g/l, pH 3.2). The wort was prepared according to the usual procedure in winemaking [5]. The strains from the collection were cultured at 26±0.5 °C in tubes with pasteurized grape must under cotton-gauze plugs. The obtained yeast inoculum was ready for use when the cell number was not less than 80 million/cm^3, budding cells not less than 30%, and the dead cells not more than 2% as assessed by light microscopy.

To obtain yeast biofilms in lab conditions, the pasteurized grape must was added with 2% yeast wort and fermented at 18±0.5 °C. By the end of fermentation when the mass concentration of sugars was not more than 4 g/l the wine material was

alcoholized to 15.3 vol.% ethyl alcohol and allowed at 18±0.5 °C until a folded light beige biofilm was formed. The physiological state of the biofilm was assessed using microscopy by the proportion of living cells not less than 50%.

The ability to form a film was determined by the rate of its growth on the surface of the fermented wort and on wine material alcoholized to 15.3 vol.%. In lab conditions, pasteurized wine-grape wort was fermented at 18±0.5 °C in 200 ml flasks with cotton gauze plugs; in micro-winemaking conditions, freshly squeezed wort was fermented at 18±0.5 °C in 3-liter glass bottles under cotton gauze plugs. Yeast wort was added to grape must (2% by volume). After the end of alcoholic fermentation, yeast film growth was monitored daily.

In lab tests of the yeast alcohol resistance, a film grown in 3-liter bottles was applied to the surface of the alcoholized wine material. Film quality and growth rate were assessed visually and by microscopy [19].

Yeasts were adapted to the 15.3 vol.% alcohol by successive frequent passages using fresh wine materials with a gradually increasing alcohol concentration as per Sayenko's method.

The selected yeast strain was tested in a winery (the Chernyavsky's farm, Evpatoria, Republic of Crimea). Oak 5003-liter wine barrels (Nos. 1, 2, and 3) were filled $^2/_3$ with different wine materials, the Aligote 50% + Sauvignon green 25% + Rkatsiteli 25% (blend 1, barrel No. 1), Sauvignon green 50% + Rkatsiteli 50% (blend 2, barrel No. 2) and Aligote wine material (barrel No. 3). Equal amounts of well-developed young yeast films grown on Aligote wine materi-al alcoholized to 15.3 vol.% in lab conditions, after checking the physiological state of the yeast, were transferred to 30% surface of the wine material in each barrel. The upper openings of the barrels were closed with cotton-gauze plugs. The barrels were allowed at 18±1 °C. During wine aging, the film quality (its appearance, the living cells at least 50%, the presence of foreign microflora) was assessed microbiologically, as well as the accumulation of aldehydes was measured. After 3-month exposure under the formed biofilm, the wine samples were taken from the barrels for biochemical analysis.

The mass concentration of volatile acids in the samples was determined as per the state standard GOST 32001-2012 ("Alcoholic products and raw materials for its production". Moscow, 2014), the mass concentration of aldehydes as per GOST 12280-75 ("Wines, wine materials, cognac and fruit spirits". Moscow, 2003), the mass concentration of residual sugars as per GOST 13192-73 ("Wines, wine materials and cognacs". Moscow, 2005), and the volume concentration of ethyl alcohol as per GOST 32029-2013 ("Alcoholic products and raw materials for its production". Moscow, 2014).

The organoleptic quality was determined by a commonly used sensory evaluation method [17].

The data were processed statistically using the Microsoft Excel program. All experiments were arranged in three biological replicates with two technical replicates of each analytical measurements. Means (M) and standard errors of means (±SEM) are calculated at a confidence level of $P = 0.95$.

Results. Figure 1 shoes the scheme we developed to find *S. cerevisiae* strains producing Jerez wines by biofilm formation on the wine material surface.

Genotyping and oenological characterization of *Saccharomyces* yeast strains. Of 143 strains involved in molecular genotyping, the ITS sequence was characteristic of the "wine" type in 102 strains (71%), and of the "sherry" type in 41 strains (29%). The *YDR379C-A* gene allele specific for sherry yeast was detected in 41 strains, and a 111 nt deletion in the adhesin gene *FLO11*

promoter typical for Sherry strains — only in 12 strains. Fifty-nine strains had a "sherry" allele in at least one of the three loci tested.

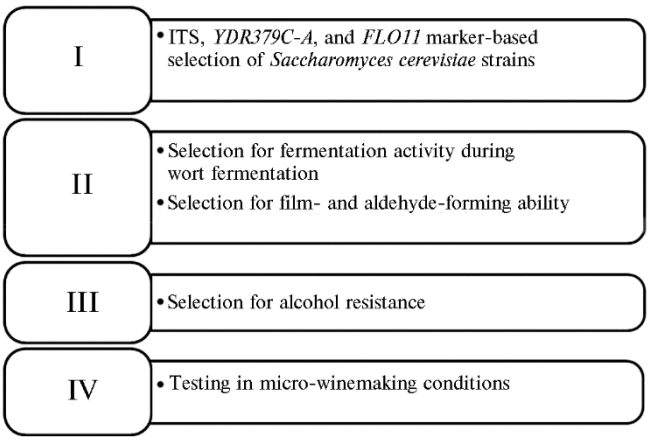

Fig. 1. The use of genetic and oenological markers to select yeast *Saccharomyces cerevisiae* candidate strains for the production of Jerez wines.

The selection of candidate strains was based on both genotyping data and the assessment of specific Jerez oenological characteristics in laboratory samples of fermented grape must (Table 1), namely, film-forming and aldehyde-forming ability and generation of characteristic properties of Jerez-type wines. Physiological, biochemical and technological studies showed that sherry yeast strains from the the Magarach Winemaking Microorganism Collection can produce 132 to 352 mg/l aldehydes during grape must fermentation phase, and it takes from 1 week to 1 month for a biofilm to appear on the fermented wort [19]. For high-quality table wine materials, the amount of aldehydes is 20-100 mg/l and often at a sensory threshold level of 40-100 mg/l [18]. This is largely due to the yeast strains with low oxidative activity. The candidate sherry strains should synthesize more than 100 mg/l aldehydes.

1. Genetic and oenological markers of *Saccharomyces cerevisiae* strains for Jerez-type wine production (n = 143, the Magarach Winemaking Microorganism Collection, Republic of Crimea)

Strain	Organoleptic quality	MCA, mg/l (M±SEM)	Film formation	
			F	R
		ITS locus		
I-31	Oxidized, salty taste	107.4±6.5	Film	66%
I-43	–	103.4±6.3	–	
I-53	Sherry tone, oxidized, salty taste	132.0±8.0	Film (ring)	66%
I-214	–	114.4±6.9	–	
I-307	Sherry tone, oxidized taste	160.6±9.7	–	
I-440	Sherry tone, oxidized, salty taste	148.7±9.0	–	
I-471	–	178.2±10.8	–	
I-527	–	123.2±7.5	–	
I-630	–	92.4±5.6	–	
I-651	–	171.6±10.4	–	
I-653	–	123.2±7.5	–	
No. 19	–	71.3±4.3	–	
No. 28	–	73.0±4.4	–	
No. 74	–	37.8±2.3	–	
No. 75	–	128.5±7.8	–	
No. 76	–	124.9±7.6	–	
		YDR379C-A locus		
I-133	Sherry tone, oxidized, salty taste	171.6±10.4	Film	100%
I-308	–	114.4±6.9	–	
I-492	Sherry tone, oxidized, salty taste	180.4±10.9	Film	100%
I-616	Sherry tone, oxidized taste	184.8±11.2	Film	66%
No. 45	–	84.5±5.1	–	
No. 49	Slight sherry tone, oxidized taste	129.2±7.8	Film	66%

Continued Table 1

No. 71	–	75.7±4.6	Film (floating islets)	33%
No. 78	Slight sherry tone, oxidized taste	154.9±9.4	Film (floating islets)	33%
No. 79	–	91.5±5.5	–	
No. 97	Oxidized taste	202.4±12.3	–	
No. 108	Oxidized taste	183.0±11.1	–	
No. 112	–	176.0±10.7	–	
	FLO11 locus			
No. 52	Sherry tone, oxidized taste	140.3±8.5	Complete flor covering	
No. 53	–	34.3±2.1	Film (floating islets)	100%
No. 90	–	74.5±4.5	–	66%
	ITS + *YDR379C-A* loci			
I-14	Aldehyde tone in the aroma, oxidized, salty taste	198.0±12.0	Film (ring)	66%
I-118	Oxidized, salty taste	162.8±9.9	Film (ring)	66%
I-137	Aldehyde tone in the aroma, oxidized taste	103.4±6.3	–	
I-280	–	149.6±9.1	–	
I-310	Aldehyde tone in the aroma, salty taste	316.8±19.2	Film (floating islets)	100%
I-340	Oxidized, slight salty taste	101.2±6.1	Film (ring)	100%
I-374	–	123.2±7.5	Film	100%
I-380	Slight bread tones in the aroma	167.2±10.1	Film	100%
I-448	Oxidized taste	123.2±7.5	Film (ring)	33%
I-515	Oxidized, salty taste	198.0±12.0	Film (floating islets)	100%
I-516	–	149.6±9.1	Film (ring)	100%
I-523	Oxidized, salty taste	85.4±5.2	Film (floating islets)	33%
I-525	Sherry tone in the aroma, oxidized, salty taste	149.6±9.1	–	
I-654	Oxidized, salty taste	132.0±8.0	Film (floating islets)	33%
No. 4	Oxidized, salty taste	67.8±4.1	Complete flor covering	66%
No. 18	–	134.0±8.1	Film (floating islets)	33%
No. 27	Oxidized taste	98.7±6.0	Film (floating islets)	33%
No. 77	–	184.8±11.2	Film (ring)	66%
	ITS + *FLO11* loci			
	No strains found			
	YDR379C-A + *FLO11* loci			
No. 46	–	109.7±6.7	Film (ring)	33%
No. 54	Sherry tone in the aroma, oxidized, salty taste	148.7±9.0	Complete flor covering	100%
No. 110	Oxidized taste	253.4±15.4	Film (ring)	100%
	ITS + *YDR379C-A* + *FLO11* loci			
I-271	Sherry tone in the aroma and in the taste	343.2±20.8	Complete flor covering	66%
No. 3	Sherry tone in the aroma and in the taste	65.9±4.0	Complete flor covering	100%
No. 23	Sherry tone in the aroma and in the taste	105.6±6.4	Complete flor covering	100%
No. 109	Slight sherry tone in the aroma and in the taste	283.4±17.2	Complete flor covering	100%
No. 111	Sherry tone in the aroma and in the taste	279.8±17.0	Complete flor covering	100%
No. 113	Sherry tone in the aroma and in the taste	176.0±10.7	Complete flor covering	100%
	Control			
I-329	Sherry tone, oxidized, salty taste	350.6±21.3	Complete flor covering	100%

Note. MCA — mass concentration of aldehydes, F — yeast biofilm on the surface, R — reproducibility. Dashes indicate the absence of the trait.

Sixteen strains carrying only the ITS sherry marker presented a high frequency of samples (75%) with active aldehyde formation and a low frequency of those (13%) capable of surface growth (from the rings on the bottle walls formed by the collection strain I-53 and up to complete biofilm formation by collection strain I-31) with 66% reproducibility. Organoleptic evaluation of lab cultured wine materials revealed aldehyde tone in aroma and oxidation in taste in four collection strains (I-31, I-53, I-307, I-440) which produced 107.4-160.6 mg/l aldehydes.

Testing for the sherry allele of only the *FLO11* locus revealed three field isolates (Nos. 52, 53, and 90) of which two (Nos. 52, 53) developed a film. For one strain (No. 52), all oenological sherry characteristics appeared (complete film formation with a 100% reproducibility, production of 140.3 g/l aldehydes, an aldehyde tone in the aroma and oxidation in the taste).

The most informative was the use of the *YDR379C-A* marker. Of the 12 strains for which we determined only this sherry locus, three collection strains (I-133, I-492, I-616) and two isolates (Nos. 49, No. 78) showed all sherry characteristics. Of these, two collection strains (I-133 and I-492) developed flor covering the entire surface of the wine material with a 100% reproducibility. Nine

strains (75%) synthesized aldehydes (more than 100 mg/l), six strains (50%) formed a biofilm on the surface (from islets to entire covering) with 33 to 100% reproducibility. Aldehyde aroma and oxidation in taste were characteristic to 58% of the samples tested.

For combination of two sherry loci, the ITS + *YDR379C-A* (19 strains) or *YDR379C-A* + *FLO11* (3 strains), the number of strains with oenological sherry characteristics increased. Of these, two strains (collection strain I-380 and isolate No. 54) formed a complete biofilm with a reproducibility of 100% and synthesized aldehydes (167.2-148.7 g/l) during alcohol fermentation phase resulting in an aldehyde tone in the aroma and oxidation taste.

Oenological examination of six strains (I-271, Nos. 3, 23, 109, 111, and 113) carrying combination of three sherry loci showed that all of them developed biofilm on the entire surface of the fermented wort during optimal periods of time, and five strains produced aldehydes (more than 100 mg/l) in the course of alcohol fermentation. Tasting laboratory samples of fermented wort revealed sherry tones in aroma and taste. According to the results of genotyping and oenological sherry characteristics, these strains are close to the collection strain I-329 (control).

Our findings indicate that the sherry ITS locus of *S. cerevisiae* can serve as a marker to select strains for high oxidative capacity. Flor developed by 11 out of 12 strains with the *FLO11* gene directly responsible for biofilm formation confirms the main role of this gene in the selection of strains capable of surface growth on wine material. Locus *YDR379C-A* can be a selection marker for several characteristics of sherry, for example, high oxidative activity and the ability to grow on the surface of wine material. The *YDR379C-A* marker also makes it possible to reveal sherry strains that, according to the ITS typing, are classified as wine strains.

Therefore, ITS, *YDR379C-A*, and *FLO11* sherry alleles are indicative of promising strains for the production of Sherry wines. Based on genotyping, six samples, the I-271 (industrial strain), Nos. 3, 23, 109, 111 and 113 (field isolates), were involved in technological tests.

Oenological characterization of strains during preparation of wine materials in micro-winery conditions. In the test, we used the I-271 strain and two isolates, No. 3 and No. 23 selected in our previous work [17]. Grape must fermentation in micro-winery conditions showed that the prepared wine materials met all the requirements for sherry-type wine materials. The strains differed slightly in fermentation activity and production of volatile acids. They fermented sugars during the optimal time and produced 12.5-13.1 vol.% alcohol, while the residual sugars (1.2-2.5 g/l) did not exceed the permissible level. We considered the latter indicator as the main one at this stage of selection, since the low fermentation activity associated with incomplete fermentation of grape must sugars could negatively affect the biofilm formation [28]. The mass concentration of volatile acids was 0.10-0.57 g/l, of aldehydes — 65.9-105.6 mg/l, with no extraneous tones in the taste and aroma.

In breeding sherry yeas, the most important features are the ability to rapidly develop a surface biofilm on the wine containing 15-16 vol.% alcohol and to synthesize aldehydes amounting at least 350 mg/l final concentration in the sherry wine material under the biofilm [21]. Acetaldehyde being 90% of all wine aldehydes is an important component of Jerez wines, mainly affecting their aromatic characteristics [22-24]. The rate of biofilm formation on the wine material and the accumulation of aldehydes during biological aging largely depend on the strain used. Basically, the formation of a sherry biofilm occurs within a period of 3 to 30 days, depending on the strain and alcohol content [25], and the acetaldehyde concentration in the wine material can reach 1000 mg/l [23, 26-28].

The yeast strains tested (No. 3, No. 23, I-271, and I-329) were similar in the rate of surface growth. It began in 8-10 days from the end of fermentation phase, and the biofilm covered the entire surface of the wine material in 13-16 days. In terms of the amount of aldehydes and organoleptic characteristics, wine materials aged under the biofilm met the requirements for biologically aged wine materials (Table 2).

2. Characterization of promising *Saccharomyces cerevisiae* strains with combination of three sherry loci (ITS + *YDR379C-A* + *FLO11*) **by flor formation and aldehyde production in a micro-winery test** (the Magarach Winemaking Microorganism Collection, Republic of Crimea)

Strain	Biofilm growth, days		Biofilm appearance in 1 month	Aldehydes, mg/l (M±SEM)	Tasting characteristic
	islets	entire surface covered			
I-271	9	15	Well-formed, thin, elastic	426,8±5,7	Sherry tone in the aroma and in the taste
No. 3	9	16	Thin, heterogeneous in density, with gaps, light beige	492,9±7,7	Slight sherry tone in the aroma and in the taste
No. 23	10	16	Well-formed, uniform, light beige	768,0±5,8	Sherry tone in the aroma and in the taste
I-329	8	15	Well-formed, folded, dark beige	506,0±8,8	Strong sherry tone in the aroma and in the taste

Alcohol tolerance. In biofilms of the yeast strains Nos. 3, 23 and I-271 developed on the wort with 12.5-13.1% ethanol and transferred to the wine material containing 16% ethanol, growth sharply slowed down followed by the yeast cell death. We applied the Sayenko's method to gradually adapt sherry yeasts to increased alcohol concentrations [26]. The collection strain I-271 adapted well and quickly formed the biofilm over the entire surface of the wine material (Fig. 2). Strains No. 3 and No. 23 at an alcohol concentration of 15.3% reduced growth activity which resulted in 70-80% surface covered. In these strains, we noted a good physiological state of the film (up to 70% of living cells), despite a decrease in growth rate [27].

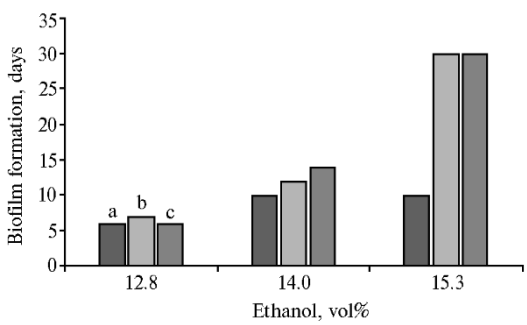

Fig. 2. Biofilm formation in promising *Saccharomyces cerevisiae* strains combining three sherry loci (ITS + *YDR379C-A* + *FLO11*) as depends on the ethanol concentration in the wine material: a — collection strain I-271, b — field isolate No. 3, c — field isolate No. 23 (a micro-winery test; the Magarach Winemaking Microorganism Collection, Republic of Crimea).

The collection strain I-271 was close to the control sherry strain I-329 in terms of alcohol resistance, biofilm formation, organoleptic parameters, accumulation of aldehydes, and the physiological state of the film cells.

Testing strains in a winery conditions. Experimental samples of Jerez-type wines were produced by biological aging under biofilm [28]. As per the chemical analyzes, biofilm growth rate, mass concentration of aldehydes, and tasting characteristics, all the blends corresponded to the sherry type wine material biologically aged for 3 months. The complete overgrowth of the biofilm on the surface of the wine materials occurred almost simultaneously: in barrels No. 1 and No. 2 — on day 12 after biofilm inoculation, in barrel No. 3 on day 10. The aldehyde concentrations after a 3-month exposure under the biofilm was 618.4 mg/l in barrel No. 1, 506.0 mg/l in barrel No. 2, and 537.7 mg/l in barrel No. 3 (5% measurement error), which corresponds to the technological instructions [21]. All wine materials were transparent, straw-colored, with a clean light aroma and sherry tones in aroma

and taste.

Based on the screening data and production tests, the collection strain I-271 can be recommended to produce sherry-type wines from both the traditionally used Aligote white grape variety and the blends of Rkatsiteli and Sauvignon green grape varieties, new for sherry wines, which opens up prospects to generate new Jerez wine brands.

Thus, our findings show that the pre-selection of *Saccharomyces cerevisiae* strains by the genetic markers ITS, *YDR379C-A*, and *FLO11* allows rapid and reliable identification of sherry strains most promising for wine production. Genotyping of 143 wine yeast strains identified strain No. I-271 with a high potential for sherry-type wine production due to alcohol resistance, formation of a complete, thin, elastic biofilm and sherry tone in aroma and taste of the wine materials.

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SEARCH FOR RYE AND WHEAT GENOTYPES WHICH ARE RESISTANT TO *Claviceps purpurea* (Fr.) Tul. AND HAMPER ACCUMULATION OF ERGOALKALOIDS IN SCLEROTIA

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Abstract

Ergot [*Claviceps purpurea* (Fr.) Tul.] is a progressive disease of rye and wheat crops. Ergot alkaloids (EA) derived from the fungus can cause severe health problems in both humans and animals. Ergot-resistant cereals are the guarantors to solve the problem. Here, we aimed to determine the EA profiles and content in sclerotia of the Kirov population of *C. purpurea* and to search for rye and wheat genotypes resistant to ergot and with no EA accumulation to be used as sources of these traits in breeding. One hundred varieties of winter rye (*Secale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) obtained from the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR) and bred at the Rudnitsky Federal Agricultural Research Center of the North-East were tested in 2017-2019 for ergot resistance by artificial inoculation of flowers with a suspension of *C. purpurea* conidia (3×10^6/ml). EA profiles and amount were estimated by thin layer chromatography on silica gel plates (Silica gel F_{254}, Merck, Germany). The metabolites were identified by co-chromatography with standards and by UV spectra (a UV-160A spectrophotometer, Shimadzu, Japan) and mass spectra (an LCQ Advantage MAX spectrometer, Thermo Finnigan, Germany). Most of the tested samples were susceptible to ergot. In wheat, only two varieties were immune (Novosibirskaya 18 and a new line T-66) and 13 varieties were relatively resistant with a lesion level of no more than 5.2 % and a sclerotia content in grain of no more than 0.3 % vs. 21.7 % and 1.5 % in the most susceptible (indicator) variety. In winter rye, immune forms were absent, in 10 varieties, the lesion varied from 5.8 % to 33.0 % and sclerotia contamination of grain from 0.3 % to 1.4 % vs. 100 % and 37.0 % in the indicator) variety). The immune and least affected varieties of rye and wheat can be used in breeding programs as genetic sources for the trait. The EA composition and amount in the fungus *C. purpurea* sclerotia were analyzed in 30 varieties of rye and wheat with different susceptibility to ergot. The EA composition was the same and consisted of ergocristine, ergotamine, and its stereoisomer ergotaminine. Only in the Kazakh wheat variety Samgau, we identified one EA, the ergotamine. The amount of EA in sclerotia varied from 0 % to 0.36 % of their weight for rye plants and from 0 to 2.40 % for wheat plants. Nine new rye populations bred at the at the Rudnitsky Federal Agricultural Research Center of the North-East and four wheat varieties do not accumulate EA. A weak negative relationship was found between the weight of a sclerotium and the EA accumulation, $r = -0.46$ ($p = 0.05$) for winter rye and $r = -0.32$ ($p = 0.05$) for spring wheat. The revealed tendency increases the biological hazard of the small and most difficult to separate fraction of sclerotia in the grain mass. No significant relationship was found between the toxicity and pathogenicity of the fungus *C. purpurea* as evidenced by the correlation between the ergot occurrence and the EA content in the rye ($r = 0.22$, $p = 0.05$). Nevertheless, data on the EA level are important for the search for immunologically and breeding-valuable genotypes that combine resistance to ergot and no EA accumulation. This trait is characteristic of the German wheat variety Epos and the new rye populations Rumba, Harmony, and Symphony.

Keywords: winter rye, spring wheat, ergot, *Claviceps purpurea*, sclerotia, resistant cultivars,

In cereals, ergot epidemiological situation caused by the fungus *Claviceps purpurea* (Fr.) Tul. (*Ascomycetes*), is recently worsening in many regions of the Russian Federation [1, 2] and abroad, especially in the Baltic republics, western Belarus, and Central Europe [3-5]. In Germany, an increase in *C. purpurea* infestation is due to larger crop area of high-yielding but more susceptible hybrid rye [6]. In Belarus, sclerotia in grain fodder consignments is of particular concern, since they are identical to rye caryopsis in shape and size which reduces the efficiency of their removal from the grain mass to 23% [7].

The disease expansion and increased severity are due to a number of reasons. A certain danger is posed by unused agricultural lands in which infectious elements of *C. purpurea* persist and accumulate. Many questions remain about modern plant growing technologies aimed at minimizing soil cultivation and other treatments. Intensifying climate change, together anthropogenic and technogenic factors, negatively impact field biocenoses. In the Kirov region, *C. purpurea* annually infects half of the sown winter grain crops. In rye biocenoses, the diseases affects 0.2-5.0% plants [8, 9]. Existing grain production technologies do not provide complete protection against ergot and the ingestion of sclerotia in seeds.

The tightening of Russian state standards (GOSTs) and foreign regulations aimes to prevent sclerotia contamination of reproduced high-quality original seeds [4, 6, 10] and to ensure the quality and safety of products. Consumption of grain and feed contaminated with sclerotia can cause poisoning for humans and animals [11-14]. Canadian researchers [15] detected up to six types of ergot alkaloids (EA) in the flour from batches of wheat grain with an extremely low (0.004%) amount of sclerotia. As per other report [16], ergot alkaloids are very stable when preparing various food products from such flour. The *C. purpurea* ability to produce EAs is genetically determined but the EA profiles and amounts depend on the host plant, geographic region and climatic factors [6, 17-20]. The most common EAs are ergometrine, ergotamine, ergocornine, ergocriptine, ergosine, and ergocristine (21, 22).

The Tatar Research Institute of Agriculture (Federal Research Center of the KSC RAS, Kazan) and the Federal Research Center Nemchinovka (Moscow Region) have focused their research on genetic protection against ergot. The gene pool of the main food crops — winter rye and spring wheat has practically not been studied for ergot resistance, especially under artificial inoculation. The EAs composition and content in the sclerotia of various grain crops and the mechanisms of plant resistance to *C. purpurea* are still poorly understood, which partly limits the progress in breeding for ergot resistance.

Genetic studies in the *Secale sereale* L.—*C. purpurea* system are difficult due to special biological traits of the pathogen and a complex procedure for the infection loading necessary to generate resistance donors. The lack of information on the phenotypic and genetic structure of populations of the genus *Claviceps* and biochemical markers of resistance are additional limitations. An important biomarker actively used in Germany is increased pollen production and pollen fertility [23]. Due to the significant influence of the environment on the pathogenesis in the *S. sereale*—*C. purpurea*, researchers note that reliable assessment of the resistance to ergot necessitates repeated testing of a genotype [23].

The total amount of EAs in sclerotia (toxicity) was shown to weakly and positively correlate with the *C. purpurea* pathogenicity to 100 new varieties of rye and wheat. Lab and field tests detected effective genetic sources of a combined plant resistance to ergot and EA accumulation in grain for use in breeding.

The work aimed to determine the total content and composition of ergot

alkaloids in the *Claviceps purpurea* sclerotia collected from winter rye and spring bread wheat plants in the Kirov region, and to identify genotypes resistant to ergot and EA accumulation.

Materials and methods. Field phytopathological testing was carried out in 2017-2019 (the Rudnitsky Federal Agrarian Scientific Center of the North-East — FASC of the North-East). The genotypes tested were 26 new diploid (×2) varieties of winter rye (*Secale cereale* L.), 20 varieties of spring wheat (*Triticum aestivum* L.) (bred at the FASC of the North-East), 34 winter rye samples and 20 spring wheat samples (accessions from the VIR world collection, Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources) [24]. The gene pool of these cereals was investigated by plant inoculation with an aqueous suspension of *C. purpurea* conidia. The conidia were isolated from sclerotia freshly collected in the Kirov region on rye and wheat plants and stored on potato-glucose agar in the working collection (FASC of the North-East). The inoculated plants were compared to control plants not inoculated with *C. purpurea*.

Immediately before inoculation, the spores were washed off the surface of a pure culture of the pathogen with distilled water. The spore concentration was adjusted to 3×10^6 conidia/ml using a Goryaev chamber [25]. The inoculum was introduced into the ovary with a syringe in the period from heading to the beginning of flowering (phases 55-61 on the Zadoks scale). Ten to fifteen plants were inoculated in 3-fold repetition. Two indicators used to characterize the varieties were the percentage of the diseased plants (prevalence) and grain contamination as the amount of sclerotia per grain sample (w%). After threshing the inoculated plants, all sclerotia were separated from the grain, weighed, and their mass ratio to the grain mass was calculated. The collected sclerotia were described biometrically by weight and size and analyzed for EA qualitative and quantitative composition. Ergot resistance of cultivars was assessed according to the scale of Miedaner et al. [26]. The disease prevalence of up to 0.5% with no more than 0.01% sclerotia correspond to high resistance, up to 1.5% and up to 0.10% — to moderate resistance, and prevalence of more than 3.0% and up to 0.3% of sclerotia per grain weight means susceptibility of the genotype.

EA profiling was performed in 30 varieties of rye and wheat, different in susceptibility to ergot (Federal Research Center PSCBR RAS, Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino). EA was extracted from 1 g of crushed sclerotia in two ways. In the first experiment, the samples were extracted three times with a 50% aqueous solution of acetone containing H_2SO_4 to create acidic conditions (pH 4.5). The bulk extract was concentrated on an IR-1M2 rotary evaporator (Khimlaborpribor, Russia) to half the initial volume. The resulting aqueous fraction was added with 25% ammonia solution to pH 9-10 and extracted 3 times with chloroform. Chloroform extracts were dried with anhydrous Na_2SO_4 and evaporated on a rotary evaporator. The second method consisted in the extraction of EAs with a mixture of chloroform and methanol (1:1).

The extracts were analyzed by thin layer chromatography (TLC) on silica gel plates (Silica gel F_{254}, Merck, Germany) in chloroform:methanol:25% NH_4OH solvent system, at 90:10:0.1 (I) and 80:20:0.2 (II). EAs were detected by absorption or fluorescence in UV light (λ = 264 nm) and after spraying the plates with Ehrlich's reagent. EAs were isolated and purified by preparative TLC on silica gel plates. Metabolites were identified by chromatography with standard samples and by UV spectroscopy (a UV-160A spectrophotometer, Shimadzu, Japan) and mass spectrometry (an LCQ Advantage MAX quadrupole mass spectrometer, Thermo Finnigan, Germany; direct sample injection into a chemical ionization chamber by a single-channel syringe pump at atmospheric pressure). The total amount of EAs in the extracts was determined spectrophotometrically in methanol

at $\lambda = 313$ nm. The calculation was carried out using the molar extinction coefficient of ergotamine ($\log \varepsilon = 3.86$). For each sample of the extract, all measurements were repeated at least 5 times.

Data were processed statistically using the methods of variance and correlation analysis. The calculation matrix included data on the disease prevalence and grain contamination with sclerotia. The significance of differences vs. the standard varieties of rye (Falenskaya 4) and wheat (Bazhenka) was estimated. The software package for statistical, biometric and genetic analysis AGROS (version 2.07) and Microsoft Office Excel were used. The EA concentrations are presented as the arithmetic mean (*M*). Confidence intervals for the EA levels (±SEM) did not exceed ±5% ($p \leq 0.05$).

Results. The rye and wheat cultivars in the control presented no symptoms of the disease or had single sclerotia. With artificial inoculation, the cultivars ranged from immune to susceptible forms. In new diploid (×2) rye populations, the prevalence varied from 14.2 (cv. Leda) to 78.5% (cv. Niobe), in diploid accessions from the VIR collection — from 5.8 (Podarok NP) to 100% (cv. Benyakonskaya 2, cv. Getera 2); grain infestation with sclerotia varied, respectively, from 0.6 (Leda) to 7.6% (cv. Gratsia) and from 0.3 (cv. Podarok NP) to 37.0% (cv. Kompus).

1. Winter rye (*Secale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) varieties resistant to *Claviceps purpurea* (Fr.) Tul. (*M*±SEM; artificial inoculation, field tests, Kirov, 2017-2019)

Variety. line, geographic origin	Ergot prevalence, %	Grain contamination with sclerotia, %
Winter rye		
Varieties from the VIR collection		
Podarok NP, Leningrad Province	5.8±0.50*	0.3±0.02*
Chulpan 2, Leningrad Province	14.2±2.80*	0.6±0.08*
Vavilovskaya NP, Leningrad Province	15.3±2.90*	1.0±0.09*
Rossiyanka 2, Leningrad Province	17.6±3.10*	0.9±0.25*
Triodis 4 — Minvak-139/09 NP, Leningrad Province	16.6±2.80*	0.6±0.09*
Krasnoyarskaya universal'naya NP, Leningrad Province	18.7±3.90*	0.8±0.19*
Average	46.6	4.9
Varieties bred at the Rudnitskii FASC of the North-East		
Leda	14.2±2.80*	0.6±0.08*
Simfoniya	19.5±4.20*	1.2±0.28*
Garmoniya	30.7±5.70*	1.2±0.29*
Rumba	33.0±6.40*	1.4±0.31*
Falenskaya 4 (standard)	40.7±8.20	3.5±0.75
Average	57.4	3.6
Benyakonskaya 2 (reference variety)	100	20.0±6.80
Kompus (reference variety)	50.0±8.80	37.0±9.90
Spring soft wheat		
Varieties from the VIR collection		
Novosibirskaya 18, Novosibirsk Province	0*	0*
Tulaikocskaya nadezhda, Samara Province	1.3±0.11*	0.1±0.01*
Kayir, Kazakhstan	1.3±0.11*	0.1±0.01*
Ul Alta Blanca, USA	1.6±0.18*	0.1±0.01*
Epos, Germany	2.1±0.52*	0.2±0.03*
Samgau, Kazakhstan	2.5±0.50*	0.2±0.03*
Average	3.7	0.2
Varieties bred at the Rudnitskii FASC of the North-East		
T-66	0*	0*
C-65	1.7±0.16*	0,1±0,01*
U-80	2.9±0.80*	0,1±0,01*
U-28	4.7±0.95*	0,2±0,03*
C-84	5.1±1.00*	0,2±0,03*
T-123	5.1±1.01*	0,2±0,03*
T-141	5.2±1.00*	0,3±0,05
Bazhenka (standard)	7.4±1.45	0,4±0,15
Average	8.1	0,27
P-57 (reference variety)	21.7±4.40	1,5±0,80

Note. NP — a low-pentose variety.

* Differences from standards (cv. Falenskaya 4 for rye and cv. Bazhenka for wheat) are statistically significant at $P \geq 0.95$.

Table 1 shows cultivars which were the least affected during 3 years of study and are immunologically valuable for rye breeding.

Miedaner et al. [6, 26] found that among four groups of rye plants (population, crossbred, synthetic, and hybrids), population varieties were 2 times more resistant to ergot. Grain contamination with sclerotia was 0.37% for the least affected population variety and 0.89% for a hybrid. There were no significant differences in the composition of EAs [26]. The most common alkaloids were ergosine, ergocristine, and ergotamine. However, their overall content had significant genotypic variability. The phenotypic manifestation of the trait is also associated with the ploidy of the genotype, as evidenced by the studies of rban et al. [3]. According to the authors' report, tetraploid (×4) genotypes which have a longer open flowering period than diploid varieties (×2) are more susceptible to ergot. It can be assumed that diploid rye populations are more promising in breeding for ergot resistance. At present, 109 varieties of winter rye are included in the State Register of Breeding Achievements in the Russian Federation, but there are only 10 hybrids and 9 varieties of the tetraploid type, the rest are diploids. In Germany, on the contrary, hybrids occupies approximately two thirds of all areas for rye cultivation [26].

The ergot resistance of spring wheat, which is mainly due to the short time and closed type of flowering, was significantly higher [25]. Nevertheless, as in rye, significant variability of immunological parameters occurred (see Table 1). In the varieties of the Rudnitskii North-East FASC, the disease prevalence varied from 0 (T-66) to 21.7% (P-57) with 0 (T-66) to 1.5% (P-57) sclerotia in grain batches; for collection accessions, the parameters ranged from 0 (Novosibirskaya 18) to 13.9% (LT-3) and from 0 (Novosibirskaya 18) to 1.3% (LT-3), respectively. The Novosibirskaya 18 variety and the new T-66 line did not develop sclerotia upon artificial inoculation with *C. purpurea* (see Table 1). The absence of sclerotia could be due to genes that control the physiological mechanisms of resistance. Thus, the Novosibirskaya 18 plants rapidly develop in the first half of ontogeny (from germination to flowering, phases 10-69 on the Zadoks scale), and belonged to the early maturing group. The stability of the T-66 line was probably due to its dwarfism and the strength of the straw, which allow the plants to avoid lodging and ensure pollen formation. We classified the varieties Tulaikovskaya nadezhda and Kayir as medium-resistant.

Among the studied winter rye and spring soft wheat gene pools, the collection accessions from Russia and abroad showed the best immunological estimates on resistance to ergot compared to the varieties bred at the North-East FANC. Immune and less affected varieties we selected under a severe infectious load can be the donors of resistance.

The biometric parameters of sclerotia varied significantly in rye and wheat varieties. Thus, the weight of a sclerotium in rye varied from 0.05 (Bereginya NP) to 0.34 g (Grafite) and averaged 0.10 g for collection accessions and 0.20 g for the North-East FANC varieties. In spring wheat, the Chinese variety Long Chun 7 and the line H-154 had the smallest (0.06 g) and large (0.15 g) sclerotia, respectively. Both in rye and in wheat, the largest sclerotia appeared in the varieties bred at the North-East FANC.

The analysis revealed the absence of EA in 9 out of 20 samples of sclerotia collected from winter rye plants and in 4 out of 10 samples from spring wheat (Table 2).

2. Ergot alkaloids in sclerotia of *Claviceps purpurea* (Fr.) Tul. developed on winter rye (S*ecale cereale* L.) and spring soft wheat (*Triticum aestivum* L.) varieties differing in resistance to ergot (*M*±SEM; artificial inoculation, field tests, Kirov, 2017-2019)

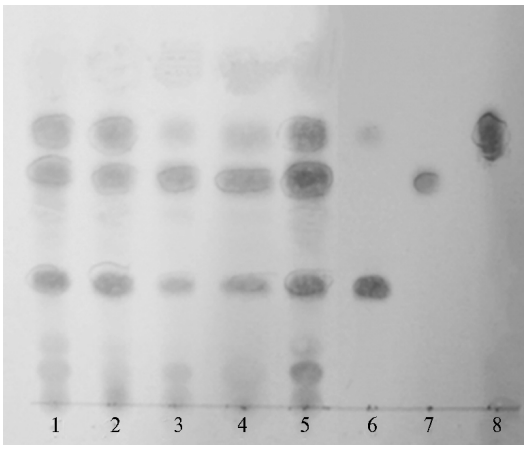
Variety, line	Geographic origin	Ergot alkaloids	
		from sclerotia weight, %	composition
Winter rye			
Vyatka 2	Kirov Province	0.22±0.009	EA, EM, EC
Falenskaya 4		0.14±0.007	EA, EM, EC
Gratsiya		0.10±0.004	EA, EM, EC
Grafit		0	
Perepel		0	
Rumba		0	
Garmoniya		0	
Simfoniya		0	
Triumph		0.04±0.002	EA, EM, EC
Nioba		0	
Leda		0.17±0.008	EA, EM, EC
Sadko		0	
Rosa		0	
Sara		0	
Amilo 2	Leningrad Province	0.14±0.006	EA, EM, EC
Yantarnaya NP		0.07±0.009	EA, EM, EC
Podarok NP		0.20±0.010	EA, EM, EC
Vavilovskaya NP		0.06±0.002	EA, EM, EC
Rushnik 2 NP		0.36±0.015	EA, EM, EC
Bereginya NP		0.06±0.002	EA, EM, EC
Spring soft wheat			
N-154	Kirov Province	0	
P-57		0.06±0.002	EA, EM, EC
C-84		0.09±0.004	EA, EM, EC
T-38		0	
T-79		0.24±0.011	EA, EM, EC
Orenburgskaya 23	Orenburg Province	0	
LT-3	Leningrad Province	0.12±0.005	EA, EM, EC
Samgau	Kazakhstan	0.14±0.006	EA
Epos	Germany	0	
Long Chan	China	0.12±0.004	EA, EM, EC

Note. EA, EM, and EC — ergotamine, ergotaminine, and ergocristine, respectively.

In other samples, the EA levels varied significantly, e.g., in rye sclerotia from 0.04 (Triumph) to 0.36% (Rushnik 2 NP) of their mass, in wheat sclerotia from 0.60 (line P-57) to 0.24% (line T-79). In our earlier studies [9], the total content of EA in rye sclerotia reached 0.90%, which may be due to excessive moisture during the sclerotia formation. Miedaner et al. [6] also emphasized the close relationship between climatic factors, the EA content and composition. Oeser et al. [18] noted that, with regard to the wide phylogenetic specialization of the biotrophic pathogen *C. purpurea*, it is reasonable to use its various strains isolated from grain taxa.

Correlation analysis revealed a negative relationship ($p = 0.05$) between the total mass of sclerotia and the content of EA (the $r = -0.46$ for rye and $r = -0.32$ for wheat). However, this trend which is important from a practical point of view, requires additional statistical proofing. Considering that small sclerotia cannot be completely separated from grain during mechanical sorting and some of them end up in seed and food grain batches [27-29], the danger of this fraction seems to be the most serious. In turn, the rye grain contamination with sclerotia was significantly (at $p = 0.05$) influenced by the ergot damage to varieties ($r = 0.70$) and the sclerotia weight ($r = 0.69$).

In 17 studied samples of sclerotia, metabolites 1 and 2 were found migrating on TLC with Rf = 0.21 (I) and Rf = 0.49 (II). They fluoresced in UV light ($\lambda = 254$ nm) and developed violet color with Erlich's reagent. The mass spectra of the metabolites were identical and had a negative molecular ion of 580



Thin-layer chromatography of extracts from sclerotia collected on different varieties of winter rye (*Secale cereale* L.) after spraying with Ehrlich's reagent: 1 — Amilo 2, 2 — Yantarnaya NP, 3 — Podarok NP, 4 — Vavilovskaya NP, 5 — Rushnik 2 NP, 6, 7 and 8 — ergotamine, ergocristine, and ergotaminine standards, respectively. Silica gel F_{254} (Merck, Germany), chloroform:methanol:25% NH_4OH (90:10:0,1).

$[M-H]^-$. The chromatographic mobility and MS/MS spectrum of metabolite 1 coincided with the ergotamine standard, and metabolite 2 with the ergotaminine standard. Therefore, the metabolites 1 and 2 were identified as the peptide ergoalkaloids ergotamine and its stereoisomer ergotaminine. In the same samples of sclerotia, metabolite 3 with Rf = 0.41 (I) was found which also fluoresced and produced violet color with Ehrlich's reagent. MS/MS of the metabolite 3 had a negative molecular ion 608 $[M-H]^-$. The chromatographic mobility and MS/MS spectrum of metabolite 3 matched the ergocristine standard. Therefore, we identified the metabolite 3 as ergocristine (Fig.).

In rye, the correlation between the ergot damage to plants and the total EA content was $r = 0.22$ ($p = 0.05$), which indirectly indicates the absence of a significant relationship between the toxicity and pathogenicity of the fungus *C. purpurea*. Nevertheless, data on the content of ergoalkaloids is important for the search for genotypes of grain crops with the lowest accumulation of EA that makes sclerotia a less dangerous mechanical impurity in the grain mass. The varieties that combine resistance to ergot damage and lower EA accumulation are of the greatest breeding and immunological value. Among them are the German variety of spring wheat Epos and new populations of winter rye Symfonia, Garmonia and Rumba. The high-yielding ergot-resistant cv. Rumba is ready for the state testing. The Garmonia population was obtained using donors of resistance to ergot and fusariosis of the ear [1].

Thus, qualitative and quantitative analysis of ergoalkaloids (EAs) upon artificial inoculated with *Claviceps purpurea* showed that in sclerotia of five wheat varieties and eleven rye varieties the EAs were ergocristine, ergotamine and its stereoisomer ergotaminine. In the Kazakh wheat variety Samgau, we identified only ergotamine. The content of EAs in sclerotia differed significantly. Varieties of winter rye and spring wheat that do not accumulate EAs in sclerotia are of practical importance, since the contamination of food and feed grains with such sclerotia does not pose a biological hazard. Genotypes that combine resistance to ergot damage with the absence of EA accumulation in sclerotia (e.g., the winter rye Rumba, Sympfoniya, Garmoniya and spring wheat Epos) are of particular breeding value as donors of these traits.

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MULTI-MYCOTOXIN SCREENING OF FOOD GRAIN PRODUCED IN RUSSIA IN 2018

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Abstract

Accumulation and analysis of data concerning mycotoxins in food grain, their co-occurrence and concentration are essential for health risk analysis and management. Mycotoxins were analyzed in 162 samples of food wheat, barley, maize, oat and rye harvested in seven Federal Districts: Central, Volga, Urals, Siberian, Far Eastern, Southern and Northern Caucasus in 2018. High-performance liquid chromatography coupled to tandem mass spectrometry was used to detect 28 analytes: regulated mycotoxins deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA), fumonisins B_1 and B_2 (FB_1 and FB_2), aflatoxin B_1 (AFL B_1), ochratoxin A (OTA), their derivatives 3- and 15-Acetyl-DON, nivalenol (NIV), fusarenone X (FUSX), HT-2 toxin (HT-2), T-2 triol, neosolaniol (NEOS), α- and β-zearalenol (α- and β-ZEL), aflatoxins B_2, G_1, G_2 (AFL B_2, G_1, G_2), sterigmatocystin STC; *Alternaria* mycotoxins tentoxin (TE), altenuene (ALT), alternariol (AOH), its methyl ether (AME); citrinin (CIT), citreoviridin (CTV), mycophenolic acid (MPA) and cyclopiazonic acid (CPA). Most wheat samples from Central, Volga, Urals and Siberian Federal Districts were positive for Alternaria toxins, while deoxynivalenol (DON) was discovered in the wheat from the Krasnodar region. ZEA, T-2 and HT-2, OTA, CIT and MPA were present in wheat samples also. FB_1 or FB_1 + FB_2 and DON (DON or DON + 15-AcDON) prevailed in corn from the Southern and the Northern Caucasus regions. MPA and NEOS were detected in a third of studied corn samples, while *Alternaria* toxins were absent. Barley from the South of Russia was mostly contaminated with T-2 and HT-2 alongside FB_1. Like wheat, most barley samples from Central, Volga, Urals and Siberian Federal Districts were positive for *Alternaria* toxins. The occurrence of *Alternaria* toxins in rye and oat samples was high regardless of region of origin. T-2 and HT-2, NEOS and CIT were detected in these samples also. However, DON was not found in any sample of barley, rye, or wheat. To the best of our knowledge, we are the first to report CTV in food grain of wheat, barley and corn from Russia. Thus, the detected mycotoxins pattern of food grain proved to depend on the crop and the grain origin. The results correlate well with reported data on fungal contamination of cereals and mycotoxins found in feed. High OTA occurrence (7.4 % of all samples) with 45 % positives over maximum level should be noted concerning safety assurance.

Keywords: food grain, wheat, barley, corn, rye, oat, mycotoxins; deoxynivalenol, T-2 and HT-2 toxins, zearalenone, fumonisins, aflatoxins, ochratoxin A, nivalenol, fusarenone X, T-2 triol, neosolaniol, zearalenols, sterigmatocystin, tentoxin, altenuene, alternariol, citrinin, citreoviridin, mycophenolic acid, cyclopiazonic acid, co-contamination, HPLC-MS/MS

Mycotoxins, the secondary metabolites of microscopic fungi are inevitable natural contaminants of agricultural products. Cases of mycotoxycoses in humans and animals have been reported in Japan, Brazil, the USA, Europe, China, the Soviet Union, and African countries [1]. Several hundreds of mycotoxins have been described. They have different toxic effects and differ in incidence and the levels in substrates. The most hazardous to human health are fusariotoxins deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA), fumonisins B_1 and B_2 (FB_1 and FB_2), as well as metabolites of *Aspergillus* and *Penicillium* fungi — aflatoxin B_1 (AFL B_1) and ochratoxin A (OTA). Many countries have established maximum allowable levels (MALs) for these mycotoxins in food grains and grain-based products. In the countries of the Customs Union, there are technical regulations for food grain [2, 3] which establish the MALs for DON in wheat and barley at 700 and 1000 μg/kg, respectively, and for T-2 toxin in any grain at 100 μg/kg. The content of ZEA in the wheat, barley and corn grain cannot exceed 1000 μg/kg, and the total amount of FB_1 and FB_2 in corn cannot exceed 4000 μg/kg. The MAL for AFL B_1 and OTA is 5 μg/kg.

In the last decade, the development of analytical methods have significantly expanded the range of detectable mycotoxins. Universal, sensitive and selective method of high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) simultaneously recognizes tens [4-6] and even hundreds [7, 8] secondary metabolites of microscopic fungi.

Depending on the structure, incidence and danger to humans, mycotoxins are conventionally assigned to i) legally regulated mycotoxins and their structural derivatives, ii) Alternaria toxins, and iii) other mycotoxins the prevalence of which in plant-derived food products has not been fully studied. Structural derivatives are usually associated with regulated mycotoxins, and since most of them also have toxic effects, group MALs or conditional tolerable daily intake (CTDI) are used as the food safety parameters. For example, in the EU countries, both AFL B_1 and also the total AFL (AFL B_1 + B_2 + G_1 + G_2) in foodstuffs are under regulation [9]. CTDI values have been established for DON and its derivatives (3-AcDON, 15-AcDON and DON-3-glucoside) [10], T-2 toxin and its derivatives (HT-2 toxin, diacetoxyskirpenol, T-2 triol and tetraol, neosolaniol NEOS, their glycosides) [11], ZEA and its derivatives (glycosides and ZEA sulfates, α- and β-zearalenols — α-ZEL and β-ZEL, etc.) [12], fumonisins (FB_1, FB_2, FB_3 and FB_4) [13]. Alternariol (AOH) and its methyl ester (AME), tentoxin (TE), altenuene (ALT), tenuazonic acid, altertoxins, and their modified forms are the common secondary metabolites of *Alternaria* fungi in grain. The HPLC-MS/MS detects both the above-mentioned and little-studied mycotoxins, e.g., moniliformin, enniatins, beauvericin, sterigmatocystin (STC), mycophenolic acid (MPA), citreoviridin (CIT), cyclopiazonic acid (CPA). etc.

The incidence and levels of mycotoxins in grain directly depends on the prevalence of the mycotoxin producers. In the Russian Federation, *Fusarium* and *Alternaria* infections of cereals are most common [14]. In Russia, the grain levels of DON, T-2 toxin, ZEA, FB_1 and FB_2 (the secondary metabolites of the *Fusarium* micromycetes) are under regulation. DON derivatives include its acetyl derivatives, nivalenol (NIV), fusarenone X (FUSX), as well as glycosides and sulfo derivatives. Their main producers are *F. graminearum*, *F. culmorum*, and *F. cerealis*, and, depending on the geographic location, strains predominantly synthesizing NIV or DON + 3-AcDON or DON + 15-AcDON are dominant, a mixed chemotype is also possible [15-17]. A study of grain harvested in 2005-2010 showed that, on average, in Russia, the contamination for DON is much lower than the maximum allowable level (MAL) [18, 19], grain from the Southern

Federal District and North Caucasian Federal District is the most contaminated [20]. These territories, along with the Far Eastern Federal District, are traditionally the areas of grain fusarium in Russia [17, 21]. However, due to climate change and the seed exchange, more aggressive pathogens are gradually moving northward. For example, *F. graminearum*, a producer of DON and ZEA, appears in grain from northwestern Russia [22]. Since 2003, in the grain-producing regions of Western Europe, *F. culmorum* has also been replaced by a more thermophilic and toxinogenic species *F. graminearum* [16, 23].

The T-2 toxin group includes HT-2 toxin, T-2 triol, diacetoxyskirpenol, NEOS and their derivatives. Their main producers are *F. sporotrichioides*, *F. langsethiae* and *F. poae*. Contamination with these mycotoxins is especially typical for oats and barley (up to 75-95%), to a lesser extent for wheat, and for corn grain the proportion of positive samples is less than 22% [24]. The mycological studies indicate the widespread prevalence of T-2 group toxin producers in grain from the Krasnodar and Stavropol Territories [25], the Central and North-Western Federal Districts [26], in the Trans-Urals (on the example of Kurgan, Sverdlovsk, Tyumen, Chelyabinsk regions) [27]. For T-2 and HT-2 toxins in oats, barley and wheat grain, the rate averaged 59% [27]. T-2 triol and diacetoxyscirpenol (DAS) were also identified. Moreover, contamination with derivatives of T-2 toxin turned out to be more typical for oats and barley than for wheat. This is consistent with data from Croatia [28] and the results obtained for the domestic food grain harvests of 2008-2010 where T-2 toxin and HT-2 toxin contamination rates were 5-18% for wheat, 0-57% for rye, 21-27% for barley, and 13-50% for oats [18].

ZEA and its derivatives are metabolites of *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense*, and *F. semitectum* [27]. In Russia, ZEA was detected in 7% of maize food grain samples in 2000-2016, with toxin levels ranging from 5 to 315 μg/kg [29]. In 2005-2010, 2-8% wheat samples, 0-39% barley samples, and 12-67% oat samples were contaminated with ZEA [18].

F. verticillioides and *F. proliferatum*, the main FB producers, mainly affect maize and sorghum [13]. FB_1 and FB_2 abundance in corn harvested in 2000-2016 in Russia exceeded 85% and 50%, respectively, and in 10% of 271 samples the FB level exceeded the MAL [30]. There is also information about the detection of FB_1 in the fodder grains of barley and wheat [31].

Secondary metabolites of *Aspergillus* and *Penicillium*, the AFL and OTA pose the highest risks to human health. During the monitoring of mycotoxins in Russian food grain in 2013-2016, three of 49 studied maize samples (6%) contained AFL B_1, with exceeding the MAL in two samples [30]. Survey of wheat, rye, barley and oats food grain harvested in 2003 and 2004 showed OTA contamination of 6%, 34%, 16% and 8% of the samples, respectively. In 2.5% of 272 samples tested, the OTA level exceeded the MAL [32]. In grain of 2012-2014 harvests, only two samples (1%) contained this toxin [20].

Alternaria metabolites are the second most abundant in cereals after fusariotoxins [33]. They exhibit an immunomodulatory effect, AOH and AME are genotoxic agents [34]. The ubiquitous abundance of *Alternaria* micromycetes in cereals in Russia was shown using samples from the republics of the North Caucasus [35], Stavropol and Krasnodar Territories, regions of the Central Federal District [36], and the Urals [37]. In wheat, oats, and barley grain from the regions of the Ural Federal District, TE, tenuazonic acid, AOH, and AME were identified.

Information on the prevalence of other mycotoxins in domestic grain is extremely sketchy. In wheat, barley and oats (56 samples in total) from the Trans-Urals, moniliformin and beauvericin were detected (12.5% and 34.0% of samples, respectively) [27]. In Russian food grains and grain-based product, the

contamination rates for STC (a biogenic precursor of AFL B_1) reached 8% at 150 μg/kg [38]. The European Food Safety Authority (EFSA) has estimated that no more than 1.5-8.0 μg/kg of STC in cereals and grain products is safe [39]. Compared to AFL B_1, STC is less toxic but much more common. Wheat from China had six times more samples with STC contamination than with AFL B_1 [40]. It is also of interest to determine the little-studied mycotoxins CIT, CPA and MPA in food grains. CIT and OTA are often detected together, both mycotoxins are nephrotoxic, and their synergistic effect is possible. Previously, a high rate of CIT and OTA co-contamination in feed was shown [41]; data on the food grain contamination are not available. MPA, a common contaminant of plant products, can reach several milligrams per kilogram. MPA has no pronounced toxic properties, but in high concentrations is an immunosuppressant [42]. CPA is cytotoxic, capable of suppressing the immune system and often found together with AFL [43].

Simultaneous measurements of regulated mycotoxins, their derivatives, secondary metabolites of *Alternaria* and poorly studied secondary metabolites of micromycetes in grain allows assessing the compliance with current safety standards, the level of co-contamination, and a range of probable mycotoxin producers. Such information will, on the one hand, allow a more complete assessment of the risks to human health caused by grain co-contamination with regulated and non-regulated mycotoxins, and, on the other hand, identify potential threats caused by a change in a potential of mycotoxicogenic species.

This paper is the first to report the abundance of contamination with 28 mycotoxins for food grain of five crops (wheat, corn, barley, oats and rye) from the Central, Southern, Volga, Ural, Siberian, North Caucasian and Far Eastern federal districts (21 subjects in total). The analytes were regulated mycotoxins deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA), fumonisins B_1 and B_2 (FB_1 and FB_2), aflatoxin B_1 (AFL B_1), ochratoxin A (OTA), their derivatives 3- and 15-Acetyl-DON, nivalenol (NIV), fusarenone X (FUSX), HT-2 toxin (HT-2), T-2 triol, neosolaniol (NEOS), α- and β-zearalenol (α- and β-ZEL), aflatoxins B_2, G_1, G_2 (AFL B_2, G_1, G_2), sterigmatocystin STC; *Alternaria* mycotoxins tentoxin (TE), altenuene (ALT), alternariol (AOH), its methyl ether (AME); citrinin (CIT), citreoviridin (CTV), mycophenolic acid (MPA) and cyclopiazonic acid (CPA). This is the first systemic assessment of food grain co-contamination with 3- and 15-AcDON, NIV, FUSX, T-2 triol, NEOS, α- and β-ZEL, TE, ALT, AOH, AME, MPA, CPA, CIT and CTV in Russia. For the first time, CTV was revealed in wheat, barley and corn food grains.

The study aimed at the analysis of contamination of the main types of food grains from the regions of Russia with regulated mycotoxins, their structural derivatives, secondary metabolites of *Alternaria* fungi, and poorly studied mycotoxins.

Material and methods. Food grain harvested in 2018 (162 samples in total) were provided by branches of the regional Centers for Hygiene and Epidemiology of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare of the Central (Tula, Tambov, Kursk, Voronezh, Lipetsk, Belgorod and Orel provinces), Southern (Krasnodar Territory and Rostov Province), Volga (Penza, Saratov, Orenburg, Samara provinces and the Republic of Tatarstan), Ural (Tyumen and Chelyabinsk provinces), Siberian (Novosibirsk, Omsk provinces and Altai Territory), North Caucasus (Stavropol Territory) and the Far Eastern (Amur Province) Federal Districts of Russia. Samples were collected according to GOST R ISO 24333-2011 (Grain and products of its processing. Sampling. Moscow, 2013) from homogeneous batches stored at grain receiving and processing enterprises. In total, 114 samples of wheat grain, 18 samples of barley, 14 samples of corn, 8 samples of oats, and 8 samples of rye were analyzed.

Mycotoxins were determined by HPLC-MS/MS using an Agilent 1100 chromatographic system and an Agilent TQ 6410 triple quadrupole mass spectrometric detector (Agilent Technologies, USA; positive electrospray ionization at atmospheric pressure with the multiple reaction monitoring MRM mode). The voltage on the capillary of the detector ion source was 4000 V, the source temperature was 100 °C, the temperature of the drying gas (nitrogen) was 350 °C; the nebulizer pressure 60 psi (4.14 bar). The analytes were chromatographically separated (a Zorbax SB-C18 column, 150×4.6 mm, sorbent particle diameter 3.5 μm; 25 °C; the gradient elution mode). Mobile phase A was water:acetonitrile (95:5), phase B was acetonitrile. Both phases are acidified with formic acid (0.1 vol.%). Gradient scheme: start at 0% B, linear increase to 95% B over 30 min, 95% B up to 36 min, linear decrease to 0% B over 1 min; column equilibration for 6 min. The total chromatography time was 43 min, the sample volume was 20 μl. Each sample was analyzed twice. The analytes were identified by the coincidence of the retention time, the detection of characteristic product ions, and the ratio of the intensity of their signals. For quantification, the external calibration method was used. For accounting the effect of the matrix on the analytical signal of T-2 triol, HT-2 and T-2 toxins, isotopically labeled internal standards were used (the [$^{13}C_{22}$]-HT-2 for HT-2 toxin and [$^{13}C_{24}$]-T-2 for T-2 triol и T-2 toxin). Mycotoxins in grains of corn, barley, rye and oats was quantified by the method of external calibration "on a pure matrix". A series of eight multicomponent standard solutions was used for calibration. In standards for solvent, 50 μl of extractant, 100 μl of mobile phase A, and 10 μl of a mixture of internal standards were added to 50 μl of the standard solution. In standards for matrix, 50 μl of the "pure" matrix extract and 100 μl of mobile phase A were added to 50 μl of the standard solution. Detection limit (DL) and quantification limit (QL) were calculated as 3S/N and 7S/N criteria, respectively, where S/N is the signal-to-noise ratio.

Samples were prepared as described (MVI 410/4-2020 "Method of multidetection of mycotoxins in grain and primary products of its processing". Approved by Rospotrebnadzor). A 100 g portion of the sample was crushed in a laboratory mill to a homogeneous state, 5.0 g of a homogeneous ground sample was placed in a 50 ml centrifuge tube added with 5 ml of acetonitrile:water (80:20 vol.%) acidified with formic acid (0.5 vol.%). Extraction was carried out for 30 min alternately on a shaker (twice for 10 min) and in an ultrasonic bath (10 min). The resulting extract was filtered, a 200 μl aliquot was diluted with 600 μl of mobile phase A, centrifuged at 4000 rpm the least, degreased with hexane if necessary. The diluted extract (200 μl) was placed to a chromatographic vial added with 10 μl of a mixture of internal standards ([$^{13}C_{24}$]-T-2 and [$^{13}C_{22}$]-HT-2) (for wheat samples). The analysis was performed in duplicate.

Results. Table 1 shows the MRM parameters, retention time, recovery rates (average values for the studied crops), detection limits (DL) and quantitation limits (QL) for mycotoxins. For calculation, the first of mother ion—daughter ion transition indicated in Table 1 was used.

Wheat grain contamination. In wheat from Russian regions, DON, T-2, and HT-2 toxins, Alternaria toxins (AOH, AME, and TE), OTA, CIT, MPA, and ZEA were more common compared to other mycotoxins (Table 2). Acetyl derivatives of DON (3- and 15-AcDON) were found only in two samples of wheat from the Amur Province. In single cases, STC (1.3 μg/kg, a sample from the Lipetsk Province), β-ZEL (366 μg/kg, a sample from the Amur Province) and CTV (56 μg/kg, a sample from the Krasnodar Territory) were detected. NIV, FUSX, NEOS, T-2 triol, AFL, FB, ALT, α-ZEL and CPA were not detected.

The main contaminants of the wheat grain from the Central, Volga and Ural federal districts were *Alternaria* mycotoxins among which TE dominated. It

was detected in 62-88% of samples. This data is well consistent with the results of Orina et al. [37, 44] for wheat harvests of 2017 and 2018 from the Ural Federal District who reported TE detection (from 2.9 to 79.9 μg/kg) in all 36 tested samples. No more than 7% and 11% of the samples we tested were contaminated with T-2 + NT-2 and DON toxins, respectively. In none sample the content of the regulated mycotoxins exceeded the MAL.

In wheat from the Siberian Federal District, TE also prevailed (91%). In 20% of the samples, we found T-2 + NT-2 and DON; the content of regulated mycotoxins, with the exception of OTA, was significantly below the MAL. OTA was detected in 4 out of 11 samples (36%), and in one sample with a more than 4-fold excess of the MAL. On average, in all regions, the rate of OTA in wheat was 6%, or 7 samples. Interestingly, three of these samples (one sample from the Krasnodar Territory and two from the Omsk Province) were co-contaminated with OTA and CIT. High co-contamination of wheat with OTA and CIT was previously noted for feed grains from the central regions of the European Russia (16 out of 30 OTA-contaminated samples contained CIT) [41].

Another feature of mycotoxin contamination in wheats from the Central, Volga, Ural and Siberian districts was the MPA rate of 3-13% (at the level ranging from 40 to 3700 μg/kg). This is partly consistent with the reports on the MPA rate < 6% at 63-1255 μg/kg wheat fodder grain [45]. This mycotoxin was not found in wheat from other regions. The cases of high MPA accumulation in food grain revealed by us (3500 and 3700 μg/kg in wheat from the Tyumen and Saratov regions, respectively) deserve attention. Previously, it was reported that violated storage conditions led to self-warming and an increase in the MPA content in sunflower seeds from 53 to 2630 μg/kg [46]. MPA does not have pronounced toxic properties, but is widely used in transplantology as a strong immunosuppressant. The standard daily therapeutic dose of MPA is about 1.5 g [47], which is two orders of magnitude lower than its content in a kilogram of the most contaminated wheat grain samples. However, given the prevalence of MPA in mass-produced foods on the Russian market [48, 49], the possibility of chronic dietary intake of this mycotoxin is of concern.

Wheat from the main grain-producing regions of Russia — the Southern Federal District and North Caucasian Federal District was less contaminated with Alternaria mycotoxins than samples from the Central, Volga, Urals and Siberian regions. The tests did not reveal alternariatoxins in grain from Rostov Province. Of them, only TE was detected (in 13% of samples from the Krasnodar Territory while in other regions its rate could reach 100%. TE was detected in three of 21 samples (14%) from the Stavropol Territory, and AME was detected in the other three samples (14%). We did not detect AOH in wheat from the Southern Federal District and North Caucasian Federal District. According to the special literature, the co-infection of *Alternaria* and *Fusarium* micromycetes can suppress the synthesis of AOH [50]. Indeed, the main contaminant of wheat from these regions is traditionally fusariotoxin DON. In samples from the Krasnodar Territory, the rate for DON was 40%, with two samples exceeding the maximum permitted levels. In one sample, along with DON, we detected ZEA, OTA, and CIT, with the OTA content almost 2 times higher than the MAL. Only 10% of wheat samples from the Stavropol Territory were DON-positive, all below the MAL. Acetyl derivatives of DON were not detected in the samples from the Southern Federal District and North Caucasian Federal District. Wheat grain from the Rostov Province (Southern Federal District) turned out to be the least contaminated (only one sample contained CIT).

1. Mycotoxin detection parameters and their characteristics in the analysis of food grain by high performance liquid chromatography–tandem mass spectrometry (an Agilent 1100 chromatographic system, Agilent TQ 6410 mass spectrometric detector, Agilent Technologies, USA)

Analyt	t_R, min	Mother ion, m/z		Daughter ions, m/z	F, V	CE, V	Degree of extraction, %	DL, μg/kg	QL, μg/kg
NIV	10.1	$[M+Na]^+$	313.3	175.3; 247.2	90	10; 2	85.1	100	200
DON	12.0	$[M+H]^+$	297.1	249.2; 203.1; 175.2	90	5; 10; 18	98.7	20	40
FUSX	13.8	$[M+H]^+$	355.2	175; 247	100	20; 4	116.3	20	40
NEOS	14.0	$[M+H]^+$	383.2	305.1; 185.1	116	2; 14	88.3	0.3	1
15-AcDON	16.2	$[M+H]^+$	339.3	261.3; 231.1	90	5;5	107.2	20	40
3-AcDON	16.6	$[M+H]^+$	339.3	231.1; 212.8; 261.3	90	5; 8; 5	97.9	10	20
FB_1	17.6	$[M+H]^+$	722.4	334.2; 352.5; 316.4	165	40; 40; 40	89.9	5	20
T-2 triol	18.1	$[M+H]^+$	405.2	303.0; 124.8;	100	12; 12	94.4	30	80
AFL G_2	18.5	$[M+H]^+$	331.1	245.2; 257.2	150	30; 30	90.7	1	2,5
FB_2	18.6	$[M+H]^+$	706.5	336.0; 354.0; 318	165	35; 30; 38	94.9	5	10
ALT	18.8	$[M+H]^+$	293.1	257.1; 239.1	45	8; 16	114.6	2	5
AFL G_1	19.5	$[M+H]^+$	329.1	243.2; 200.0; 283.0	135	25; 46; 20	92.9	0.5	1
AFL B_2	19.5	$[M+H]^+$	315.3	287.0; 259.1; 231.2	135	22; 30; 30	92.4	0.5	1
AFL B_1	20.5	$[M+H]^+$	313.2	128.0; 241.1; 285.2	135	80; 35; 20	94.3	0.5	1
HT-2	20.6	$[M+Na]^+$	447.3	345.2; 285.2	190	16; 16	90.3	2	5
$[^{13}C_{22}]$-HT-2	20.6	$[M+Na]^+$	469.2	362.0	150	30	–	–	–
AOH	21.1	$[M+H]^+$	259.1	128.0; 185.1	153	40; 30	110.7	2	5
TE	21.1	$[M+H]^+$	415.2	132.0; 312.2	107	42; 8	113.6	0.5	1
α-ZEL	22.4	$[M+H]^+$	303.2	285.2; 267.0	110	5; 10	109.2	4	10
MPA	23.6	$[M+H]^+$	321.1	303.2; 207.1	73	4; 16	101.5	4	10
β-ZEL	24.0	$[M+H]^+$	303.2	285.2; 267.0	110	5; 10	118.3	4	10
CTV	24.1	$[M+H]^+$	403.2	285.0; 297.0;	45	10; 10	94.7	4	10
CIT	24.8	$[M+H]^+$	251.2	233.1; 205.0; 115.2	93	16; 24; 52	91.5	2	3
T-2	24.8	$[M+Na]^+$	489.3	245.1; 387.1; 327.2	165	24; 17; 20	99.2	0.5	2
$[^{13}C_{24}]$-T-2	24.8	$[M+Na]^+$	513.5	344.2	190	20	–	–	–
OTA	25.5	$[M+H]^+$	404.2	239.1; 358.2; 221.0	105	20; 10; 34	96.1	0.5	1
ZEA	26.8	$[M+H]^+$	319.2	185.0; 283.2; 301.2	90	22; 5; 5	104.6	2	5
AME	26.8	$[M+H]^+$	273.1	258.0; 230.0	156	24; 30	108.6	2	5

Continued Table 1

STC	28.2	$[M+H]^+$	325.1	281.1; 310.1	120	35; 22	108.5	1	2
CPA	28.5	$[M+H]^+$	337.2	196.1; 182.1	45	16; 12	82.8	30	80

N o t e. DON — deoxynivalenol, ZEA — zearalenone, FB_1, FB_2 — fumonisins B_1 and B_2, AFL B_1 — aflatoxin B_1, OTA — ochratoxin A, 3-AcDON and 15-AcDON — 3- and 15-Acetyl-DON, NIV — nivalenol, FUSX — fusarenone X, NEOS — neosolaniol, α- and β-ZEL — α- и β-zearalenol, AFL B_2, G_1, G_2 — aflatoxins B_2, G_1, G_2, STC — sterigmatocystin, TE — tentoxin, ALT — altenuene, AOH — alternariol, AME — methyl ether of AOH, CIT — citrinin, CTV — citreoviridin, MPA — mycophenolic acid, CPA — cyclopiazonic acid. Positive electrospray ionization at atmospheric pressure in multiple reaction monitoring (MRM) mode; t_R — retention time, F, V — fragmentator voltage, CE, V — collision cell voltage, DL — detection limit, QL — quantification limit. For the degree of extraction, the average values for the studied crops are given. Dashes indicate that internal standards were added to the prepared sample before analysis.

2. Rates and levels of mycotoxins in wheat food grain from Russian regions (2018, $n = 114$; HPLC/MS-MS)

Region	Contaminated samples/total number of samples (%)	Level (min-max; averaged), μg/kg (rate, %)									
		DON	ZEA	T-2	HT-2	OTA	CIT	AOH	AME	TE	MPA
CFD	19/29 (66 %)	100 (3 %)	10 (3 %)	4 and 17 (7 %)	35 and 45 (7 %)	0.4 (3 %)	–	< QL-13; 11 (21 %)	< QL-10; 6 (21 %)	2-37; 9 (62 %)	40 (3 %)
VFD	16/19 (84 %)	120 and 370 (11 %)	16 (5 %)	15 (5 %)	5 and 45 (11 %)	–	–	< QL-10 (16 %)	< QL-21 (21 %)	1-90; 11 (74 %)	3700 (5 %)
UFD	7/8 (88 %)	–	–	–	–	2.9 (13 %)	–	–	–	5-49; 14 (88 %)	3500 (13 %)
SiFD	10/11 (91 %)	180 and 250 (18 %)	–	2 and 3 (18 %)	5-70; 34 (27 %)	0.8-22; 7.8 (36 %)	15 и 97 (18 %)	< QL (9 %)	-	6-83; 27 (91 %)	380 (9 %)
Amur Province (FEFD)	3/3 (100 %)	430 and 530 (67 %)	–	5 (33 %)	13 (33 %)	–	–	26 (33 %)	< QL (33 %)	5-90; 38 (100 %)	–
Krasnodar Territory (SFD)	8/15 (53 %)	120-1270; 500 (53 %)	5 (7 %)	–	5 and 8 (13 %)	9 (7 %)	118 (7 %)	–	–	5 and 6 (13 %)	–
Rostov Province (SFD)	1/8 (13 %)	–	–	–	–	–	2.5 (13 %)	–	–	–	–
Stavropol Territory (NCFD)	6/21 (29 %)	100 and 730 (10 %)	–	–	–	–	–	–	< QL-20 (14 %)	7-197; 72 (14 %)	–

N o t e. DON — deoxynivalenol, ZEA — zearalenone, OTA — ochratoxin A, CIT — citrinin, AOH — alternariol, AME — methyl ether of AOH, TE — tentoxin, MPA — mycophenolic acid. See Table 1 for detection protocol. If the number of mycotoxin-contaminated samples was less than 3, the analyte content in the sample is indicated instead of the concentration range and average. DL — detection limit, QL — quantification limit; a dash means that mycotoxin was not detected (the content is below DL, < DL). CFD — Central Federal District, VFD — Volga Federal District, UFD — Ural Federal District, SiFD — Siberian Federal District, FEFD — Far Eastern Federal District, SFD — Southern Federal District, NCFD — North Caucasus Federal District.

In the world, data on mycotoxin abundance in wheat grain vary considerably. It should be noted that in Russia, wheat is less DON-contaminated compared to other leading grain exporters [51]. In the EU countries, the rate for DON in wheat crops of 2005-2012, according to Alexander et al. [10], ranged from 60 to 100%, and the maximum content reached 4130 μg/kg. In durum wheat from Italy (n = 74), the contamination rate was 16% for DON at 48-2267 μg/kg, 8% for T-2 and HT-2 toxins at 10-149 μg/kg, 31% for AOH at 8-121 μg/kg, and 26% for AME at 9-48 μg/kg [52]. In 2001-2010, in winter wheat from Germany, AOH and AME reached 832 μg/kg with the rate ranged from 0-77% and 905 μg/kg at 0-33% [53].

Corn grain contamination. Mycotoxins were studied in 14 samples of corn grain (Table 3), 11 samples from the Southern Federal District and North Caucasian Federal District, two from the Central Federal District, and one from the Volga Federal District. The last three samples were slightly contaminated, FB_1 40 μg/kg + NT-2 7 μg/kg and FB_1 220 μg/kg, or ten times lower than the MALs. In grain from the Southern Federal District and North Caucasian Federal District, the main contaminants were FB_1 or FB_1 + FB_2, DON or DON + 15-AcDON. ; approximately a third of the samples contained MPA and NEOS; two out of 11 samples were co-contaminated with T-2 and HT-2 toxins. The rate of mycotoxins in descending order was as follows: FB (with FB_1 prevailing) > DON and 15-AcDON > NEOS, MPA > T-2 and HT-2 toxins > ZEA, OTA, CIT, CTV.

FBs are the main mycotoxin of corn grain in Russia [30]. According to the FB levels, all the studied samples met the requirements of the regulation. In one sample from the Krasnodar Territory, OTA (13 μg/kg) exceeded the permissible level. The relatively high incidence of NEOS in corn is consistent with data from Spain [54], while no HEOS was detected in samples from Africa, Japan [55] and Brazil [56]. The main trichothecenes of group A, T-2 and HT-2 toxins, were detected in 18% of the samples. For comparison: in corn grain from Croatia (n = 71), the rate for these toxins was 27% (the total content varied from 15 to 332 μg/kg) [28]. Along with FB and DON, T-2 toxin contaminates domestic feed grain of corn [57, 58]. We did not detect Alternaria metabolites in corn, which may be due to the low susceptibility of corn kernels to infection because of a specific structure of the ear [36]. Several publications are in line with these data. For example, less than 7% of corn food grain samples from the southern Brazil contained AOH, AME, TE [56], the rate for AOH and TE in the samples from Serbia did not exceed 10%, while for AME and tenuazonic acid, it was 40 and 35%, respectively [59]. In corn fodder grain from the Central Federal District, as per Kononenko et al. [57], the rate for AOH was 13.3%.

Contamination of barley, oats, and rye grain. In these samples, mycotoxicological analysis also revealed the dependence of the detected grain contaminants on the region of crop growth (see Table 3). The number of studied samples in these species was less than for wheat and corn, therefore, we considered the conclusions about the prevalence of mycotoxins in barley, oats and rye from different regions as indicative. Nevertheless, the results obtained are in good agreement with each other. The main contaminants of grain in the Central, Volga, Urals and Siberian regions were T-2 and HT-2 toxins (20-50%) and secondary metabolites of Alternaria, primarily TE (33-100%). Among Alternaria toxins, ALT accumulation in a barley sample from the Altai Territory reached 15 μg/kg (the only case of this toxin in the studied grain samples). The predominant contamination of oat grain with T-2 and HT-2 toxins in combination with Alternaria mycotoxins corresponds to the incidence of fungal infections caused by the producers [60].

3. Rates and levels of mycotoxins in corn, barley, oats, and rye food grain from Russian regions (2018, HPLC/MS-MS)

Region	Contaminated samples/total number of samples (%)	Level (min-max; averaged), μg/kg (rate, %)														
		DON	15-AcDON	ZEA	T-2	HT-2	NEOS	OTA	CIT	FB_1	FB_2	AOH	AME	TE	CTV	MPA
Corn (n = 14)																
SFD + NCFD	11/11 (100 %)	50-950; 407 (55 %)	14-36; 25 (27 %)	34 (9 %)	25 and 67 (18 %)	41 and 194 (18 %)	1-21; 6 (27 %)	13 (9 %)	6 (9 %)	30-1560; 370 (91 %)	40-170; 97 (27 %)	–	–	–	57 (9 %)	10-95; 39 (27 %)
CFD + VFD	2/3 (66 %)	–	–	–	–	7 (33 %)	–	–	–	40 and 220 (66 %)	–	–	–	–	–	–
Barley (n = 17)																
CFD	6/6 (100 %)	–	–	–	2-7; 4 (50 %)	5-10; 8 (50 %)	–	–	–	–	–	< QL-135; 72 (67 %)	–	< QL -5; < ПКО (67 %)	–	–
VFD + UFD + SiFD	5/5 (100 %)	–	–	–	6 (20 %)	–	–	–	2 (20 %)	–	–	–	–	< QL -23; 11 (60 %)	–	–
SFD + NCFD	6/6 (100 %)	–	–	–	2 (17 %)	5-34; 15 (50 %)	–	11 (17 %)	–	< QL and 20 (33 %)	–	24 (17 %)	5 (17 %)	5 (17 %)	10 (17 %)	–
Rye (n = 8)																
CFD	2/3 (66 %)	–	–	–	2 (33 %)	6 (33 %)	–	–	–	–	–	5 (33 %)	< QL (33 %)	8 and 15 (66 %)	–	–
VFD + UFD + SiFD	1/3 (33 %)	–	–	–	–	–	–	5 (33 %)	–	–	–	–	–	20 (33 %)	–	–
SFD + NCFD	1/2 (50 %)	–	–	–	–	–	–	–	2.5 (50 %)	–	–	–	–	15 (50 %)	–	–
Oats (n = 8)																
CFD + VFD + SiFD	4/4 (100 %)	–	–	–	2 and 35 (50 %)	34 (25 %)	8 (25 %)	–	3 (25 %)	–	–	< QL (25 %)	< QL (25 %)	9-86; 27 (100 %)	–	–
SFD + NCFD	4/4 (100 %)	–	–	–	–	–	–	< QL (50 %)	–	–	–	6 (25 %)	–	2-38; 14 (75 %)	–	–

Note. DON — deoxynivalenol, 15-AcDON — 15-Acetyl-DON, ZEA — zearalenone, NEOS — neosolaniol, OTA — ochratoxin A, CIT — citrinin, FB_1, FB_2 — fumonisins B_1 и B_2, AOH — alternariol, AME — methyl ether of AOH, TE — tentoxin, CTV — citreoviridin, MPA — mycophenolic acid. See Table 1 for detection protocol. If the number of mycotoxin-contaminated samples was less than 3, the analyte content in the sample is indicated instead of the concentration range and average. DL — detection limit, QL — quantification limit; a dash means that mycotoxin was not detected (the content is below DL, < DL). CFD — Central Federal District, VFD — Volga Federal District, UFD — Ural Federal District, SFD — Siberian Federal District, FEFD — Far Eastern Federal District, SFD — Southern Federal District, NCFD — North Caucasus Federal District.

The samples from the Southern and North Caucasian regions differed in the mycotoxin profiles depending on the crop. FB_1 in two samples out of 6 studied was distinctive for barley. Cases of FB_1 in barley food grain were reported in Tunisia (46 μg/kg, one of 31 samples) [61] and in Poland (one of 8 samples) [62]. Monitoring of mycotoxins in the fodder grain of barley harvested in 2004-2014 from the Central, Southern and North Caucasian regions revealed the FB_1 rate of 19-79% [31]. Moreover, there are cases of FBs in wheat fodder grains that are atypical for food grains, namely 6% of samples harvested in 2017 contained FBs (from 75 to 1990 μg/kg) [63]; the rate of positive fodder wheat samples from 2004-2014 harvests in southern Russia reached 50% [31]. It is noteworthy that the studied samples of barley, oats, and rye did not contain DON even in trace amounts. These data are consistent with the results of long-term monitoring. From 2009 to 2016, we tested 50 samples of rye, 28 samples of oats from the Volga, Urals, and Siberian regions, and 12 samples of barley from the Central Federal District. DON was not found, while according to Gavrilova et al. [44], the rate for DON in barley from the Ural region was 20%, and we also detected NIV. One of the studied samples of barley from the Krasnodar Territory did not meet the safety requirements for OTA (11 μg/kg).

4. Examples of co-contamination with mycotoxins of food grain harvested in the Amur Province (Far Eastern Federal District,)

Crop, year	Level, μg/kgкг											
	DON	3-AcDON	15-AcDON	ZEA	β-ZEL	T-2	HT-2	OTA	CIT	STC	AOH	TE
Wheat, 2018	430	71	36	–	–	–	–	–	–	–	26	17
Wheat, 2018	530	14	–	–	366	5	13	–	–	–	–	90
Barley, 2018	2830[a]	65	54	–	–	–	–	–	–	–	40	–
Oat, 2016	650	5	–	180	-	2	12	9[a]	30	120	No data	

N o t e. DON — deoxynivalenol, ZEA — zearalenone, β-ZEL — β-zearalenol, OTA — ochratoxin A, CIT — citrinin, STC — sterigmatocystin, AOH — alternariol, TE — tentoxin. See Table 1 for detection protocol. A dash means that mycotoxin was not detected (the content is below the detection level DL, < DL). [a] — exceeding the maximum allowable level (MAL).

From the point of view of the diversity and the rate of mycotoxins, grain from the Far Eastern Federal District is of particular interest. Table 4 shows the profiles of three individual grain samples of food wheat and barley harvested in 2018, most clearly reflecting the multiple mycotoxin contamination observed in the region. In addition, there is one sample of oats harvested in 2016 which attracted attention during our long-term monitoring due to a variety of mycotoxins. All these samples were from the Amur Province. These data indicate that the regional conditions are favorable for grain infection with micromycetes and toxin production. For example, only the samples from this region, along with DON, contained both acetyl derivatives of DON (3- and 15-AcDON) and β-ZEL. Co-contamination with eight mycotoxins was shown for a sample of oats harvested in 2016, in particular, OTA + CIT and STC were detected.

Gagkaeva et al. [21] drew attention to the high infection rate of spring wheat and barley in the Amur Province in 2019. They found DON (912-13343 μg/kg), 3-AcDON (0-293 μg/kg), 15-AcDON (19-179 μg/kg, 3-glucoside DON (98-3803 μg/kg), ZEA (92-3670 μg/kg), and moniliformin (5-218 μg/kg). AOH and AME, T-2 and HT-2 toxins in grain were at the level of tens of micrograms per kilogram. APL, OTA, STC, CPC, MPA, NEOS, DAS, and FUSX were not detected. Abundant 3-AcDON in grain is peculiar to the Far Eastern Federal District. According to the available data [21], the level of the 3-AcDON-producing *F. graminearum* DNA in grain from the Amur Province was on average 1.1-1.3 times higher than that of the 15-AcDON-producing genotype.

In our survey, in several cases, STC, β-ZEL and CTV occurred in grain samples. It should be noted that we report the contamination of food grains with

citreoviridin in the Russian Federation for the first time. This mycotoxin was detected in samples from the Krasnodar Territory (56 μg/kg in wheat and corn and 10 μg/kg in one barley sample). The rate for CTV in cereals in the Krasnodar Territory was 12%. It is known that CTV producers are micromycetes of the genera *Aspergillus* and *Penicillium*, mainly *P. citreonigrum* traditionally found in rice. For example, the toxin level in rice from Brazil reached 97 μg/kg [64], which is comparable to the levels in the samples we studied. CTV accumulated in the body have a pathological effect on the central nervous system [65]. STC was found in two grain samples — in wheat from the Lipetsk region (1.3 μg/kg) and rye from the Saratov region (0.1 μg/kg). According to the reports, the rate of STC in wheat, rye and corn grain in countries having temperate climate reached 7%; STC was more abundant in barley (up to 44% of samples) and oats (up to 57% of samples) [38]. We detected β-ZEL at 366 μg/kg in one wheat sample from the Amur Province. Structural analogs of ZEA, the α-ZEL and β-ZEL were rarely detected in grain. There are no systematic data on these mycotoxins in special publications.

Among the studied 28 mycotoxins, we did not find NIV, FUSX, T-2 triol, α-ZEL, APL and CPA. According to data from Italy [66], Poland [62] and the Czech Republic [67], the incidence of these mycotoxins in wheat does not exceed 12%. NIV is more likely to contaminate oats and barley [68]. In the present study, we did not detect NIV and FUSX in any grain tested. Fusariotoxins T-2 triol and α-ZEL are also rarely detected, while APL is more abundant in grain from countries with subtropical and tropical climates, e.g., Syria [66] and African countries [69]. However, it should be borne in mind that the increase in average annual temperatures and frequent summer droughts in the countries of Southern Europe give grounds for unfavorable forecasts of *Aspergillus flavus* infection of maize and, therefore, APL accumulation [70]. It can be assumed that such a scenario is possible in the Southern Federal District and North Caucasian Federal District of the Russian Federation. CPA also refers to metabolites of *Penicillium* and *Aspergillus*, characteristic of the tropical and equatorial zones. The reports describe cases of co-detection of APL and CPA in food products from hot countries, for example, in corn [71]. For CPA, a low abundance in fodder corn, barley and wheat grain was show in the Russian Federation (3 out of 276 samples, or 1.1% at 50-80 μg/kg) [72].

Thus, in Russia in 2018, the main mycotoxins in food wheat grain were fusariotoxins deoxynivalenol (DON), zeralenone (ZEA), T-2 and HT-2 toxins and Alternaria metabolites tentoxin (TE), alternariol (AOH) and its methyl ester (AME). Ochratoxin A (OTA), citrinin (CIT) and mycophenolic acid (MPA) were also identified. In wheat from the Central, Volga, Ural and Siberian federal districts, alternariatoxins prevailed, primarily TE the rate of which varied from 62 to 91%. More than half of the samples from the Krasnodar Territory contained DON (in two of the 15 samples, it exceeded the MAL), the rate for TE was 13%. Among the regulated mycotoxins, along with DON, 7 out of 115 samples (6%) contained OTA, exceeding the MRL in two samples. Most of the wheat samples contaminated with OTA were from the Siberian Federal District. Mycotoxins were not detected in 61% of wheat samples from the North Caucasian Federal District and 87% of samples from the Rostov Province. In other regions, more than half of the samples were contaminated. DON exceeded the MAL in three samples (2.6%), OTA in two samples (1.7%). In corn grain from the Southern Federal District and North Caucasian Federal District, the main contaminants were fumonisins B_1 and B_2 (FB_1 or FB_1 + FB_2) and DON (DON or DON + 15-AcDON). A third of the samples, were MPA- and NEOS-positive. ZEA, T-2 and HT-2, OTA, CIT, and CTV were rarely found, Alternaria toxins were not detected. All the studied samples met the safety requirements, with the exception of sample from the Krasnodar

Territory with OTA exceeding the MAL. Among fusariotoxins, the barley grain from the southern regions of Russia contained T-2, HT-2, and FB_1. Alternaria toxins were more characteristic of the Central, Volga, Urals, and Siberian regions. In several cases, CIT, CTV and OTA were found, the latter exceeding the MAL. In rye and oat grain, regardless of the region, alternariatoxins, mainly TEs, prevailed compared to other mycotoxins. We also identified T-2 and HT-2 toxins, NEOS and CIT. In one rye sample, OTA did not meet the safety requirements. The barley, oats and rye grain was not contaminated with DON even in trace amounts. Our data on CTV contamination of food corn, barley and wheat in the Russian Federation nave been obtained for the first time. The survey traced the dependence of the mycotoxin profiles on the crop and the region of cultivation. The high rate of OTA-positive samples and OTA excess of the MAL in 45% of food grain are of concern.

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COPPER ION INDUCED PRODUCTION OF ROSMARINIC ACID IN LEMON BALM (*Melissa officinalis* L.) SEEDLINGS

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Abstract

Rosmarinic acid (RA), one of the most important active ingredients of lemon balm (*Melissa officinalis* L.), exhibits antiviral, antibacterial, antioxidative and anticancer properties. Furthermore, it can improve functionality in baking process. Cu acts as a cofactor of several proteins and plays a key role in photosynthesis, respiration, lignin synthesis, response to oxidative stress and cell wall metabolism, but can be toxic to plants in high concentrations. We hypothesized that abiotic stresses, as one of the external factors inducing the defense mechanism of plants, may contribute to the production of secondary metabolites, especially RA, in representatives of the *Lamiaceae* family. In current study, RA accumulation, expression of tyrosine aminotransferase gene (TAT), contents of flavonoid and anthocyanin as well as antioxidant enzymes activities were investigated in 45-day-old *M. officinalis* seedlings after treatment with different concentrations of Cu^{2+} (0, 5, 10, 20, and 30 μM). Samples were collected and analyzed after 8 and 16 hours of treatment. Lower concentrations of Cu^{2+} positively affected RA accumulation at both aforementioned treatment times, which is consistent with the increase in TAT gene expression profile. Flavonoid, anthocyanin and soluble protein contents of the seedlings significantly decreased (except at 20 and 30 μM Cu^{2+}-treated seedlings after 8 hours). RA content and expression of TAT gene decreased significantly at the highest concentration of Cu^{2+} for 16 hours. Concurrently, elevated levels of superoxide dismutase and peroxidase activities were measured in these seedlings. Latter can indicate that lower concentrations of Cu^{2+} cause oxidative stress. Reactive oxygen species (ROS), which act as signal molecules, are accumulated and due to their positive effects on the expression of TAT gene more RA is produced. In contrast, at the highest concentration of copper ions, ROS suppressed TAT gene expression and prevented the degradation of the gene product.

Keywords: antioxidant enzyme, superoxide dismutase, catalase, peroxidase, *Melissa officinalis*, rosmarinic acid, flavonoids, anthocyanins, tyrosine aminotransferase.

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, and it has been identified from lemon balm (*Melissa officinalis* L.) as one of the major active phenolic compounds [1, 2]. It has shown various biological activities such as anti-inflammatory[3], antiviral [4], antibacterial, antioxidative [5, 6], anticancer [7] and antiallergic activities [8]. Furthermore, RA as a potential

enhancer can provide functional properties to bakery products [9]. RA is biosynthesized through two different pathways in plants. Phenylpropanoid pathway, which is considered as the main biosynthesis pathway, starts with activation of phenylalanine ammonia-lyase (EC 4.3.1.24) (PAL) enzyme and uses phenylalanine amino acid as its substrate. The second pathway is initiated with tyrosine aminotransferase (TAT) reaction that uses tyrosine as its substrate [1]. Previous reports have shown that RA production can be changed in presence of carbohydrates [10] as well as by yeast extract, Ag^+ and methyl jasmonates [11-13]. Studies in *Salvia miltiorrhiza* hairy root have shown that treatment with Ag+/yeast increases TAT activity and decreases PAL activity [13]. It was demonstrated exposure of *Phyllanthus tenellus* with Cu^{2+} increased PAL activity in the leaves [14]. Similarly, a rise in PAL activity was observed in of *Camellia sinensis* leaves in presence of copper, mercury and nickel [15].

Copper, essential microelements for plant growth and development, acts as a cofactor of several proteins such as plastocyanin (PC), Cu/Zn superoxide dismutase (Cu/Zn SOD) [16], cytochrome c oxidase [17] and plays key roles in photosynthesis, respiration, lignin synthesis, responding to oxidative stresses and cell wall metabolism [18]. This element, along with other microelements, is responsible for plant resistance to diseases [19]. Although copper is highly present in nature and needed for plant growth, its high concentrations can lead to toxicity in plants [20]. Plants exposed to heavy metal stress often encounter oxidative stress and consequently a high production of reactive oxygen species ROS [15]. Plants prevent damages caused by ROS through antioxidant systems consisting of both non-enzymatic (ascorbic acid, glutathione, tocopherol, flavonoids, etc) and enzymatic reactions (catalase, peroxidase, superoxide dismutase, glutathione, and ascorbate peroxidase and reductase) [21]. Phenolic compounds play important roles in color-stabilizing mechanism [22] and defending against pathogens attacks [23] and ultraviolet ray [24] in plants. Furthermore, they are used as indicators to investigate stresses and scavenger of free radicals [25, 26].

We hypothesized abiotic stresses as one of external factors inducing plant defense mechanism can promote the production of secondary metabolites and particularly RA in *Lamiaceae* family. The aim was to treat lemon balm seedlings with different copper concentrations and to measure the amount of RA and to analyze related plant defense mechanisms such as flavonoid and anthocyanin content, and antioxidant enzymes activities. We also aimed to measure TAT gene expression as one of important genes in RA biosynthesis pathway in *M. officinalis* seedlings.

Materials and methods. F_1 seeds of lemon balm (*M. officinalis*) were obtained from Pakanbazr (Esfahan, Iran). Methanol (HPLC grade) and orthophosphoric acid were obtained from Merck (Germany). Water (HPLC grade) was provided by membrane purification system. External standard of RA ($C_{18}H_{16}O_8$, MW = 360 $g \cdot mol^{-1}$) and other analytical reagents materials were purchased from Sigma (United States).

The seeds were treated with 2% sodium hypochlorite solution and rinsed three times with sterile distilled water. These seeds were planted in MS medium (Murashige and Skoog, 1962) with 0.8% (w/v) agar, and transferred to dark incubator (relative humidity 55±5%; 28±2 °C) for two weeks. Then, plants were transferred to 16:8 hour (Light:Dark) at 30±2 °C. The 45-day-old seedlings were collected from the medium and washed carefully with sterile distilled water and transferred to liquid MS medium. The medium was supplemented with the final Cu^{2+} concentrations of 0 (control), 5, 10, 20 and 30 μM by using $CuSO_4 \cdot 5H_2O$ salt. The treatment was applied for 8 and 16 hours as recently reported [27]. Treated

seedlings were harvested and rinsed with sterile distilled water for several times to remove the surface ions. Treated seedlings were divided into two groups: one was dried in the shade (for three days) and used to measure the RA concentration; the other group was rinsed, dehydrated and frozen in liquid nitrogen, and kept at –80 °C until measuring flavonoid and anthocyanin contents, antioxidant enzymes activity and analyzing TAT gene expression level.

RA identification and quantification was performed as recently reported [27, 28]. In brief, 0.1 g of dried sample was ground into powder and mixed with 25 mL ethanol/water (30:70 v/v) solution, then it was sonicated for 10 min and centrifuged at 4500 rpm for 5 min at 4 °C. The supernatant was transferred to new collection tube and its volume was increased to 50 ml by adding sterile distilled water. The extract solution was filtered with 0.2 μM single-use syringe filter, before injecting into column of High Performance Liquid Chromatography (HPLC, ZORBAX SB-C18 column, Agilent 1100 series, USA). The mobile phase containing 40% solvent A (orthophosphoric acid in water, 1.0% v/v) and 60% solvent B (orthophosphoric acid in methanol, 1.0% v/v), was run at 1.0 ml/min at room temperature. Identification of RA was achieved by comparing retention time of each sample with the standard, which was acquired at 330 nm.

RNX plus™ kit (Cinnagen, Iran) was used to extract total RNA as stated by manufacturer's protocol. The quality and quantity of extracted RNA were assessed by 1% agarose gel and spectrophotometer apparatus (Varian cary 50, Australia), respectively. Finally, 200 $U \cdot \mu l^{-1}$ M-MuLV Reverse Transcriptase (Fermentas, EP0441, USA), Oligo(dT)20 Primer (Fermentas, SO131, USA), dNTP (1 mM of ecah) and 20 U RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA) were used to synthesize cDNA at 42°C for 120 min.

TAT gene primers (Forward primer: 5′-CCG CTA CTT CGA TCT TCA TCC-3′ and reverse primer: 5′-CCA TTG GAA CAA AAG GGT TCG-3′) were designed by Oligo Primer Analysis Software v. 7 (https://www.oligo.net/downloads.html) in reference to available mRNA sequence of Tyrosine aminotransferase gene in *Melissa officinalis* (Gene Bank Accession No. JN863949). TAT gene was amplified by *Taq* DNA polymerase (Qiagen, Germany) with an initial denaturation for 4 min at 94 °C, followed by 30 cycles amplification (1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C) and a final extension at 72 °C for 10 min using thermocycler (Eppendorf 5331 Mastercycler Gradient PCR, Germany).

To assess TAT gene expression levels in the presence of different Cu^{2+} concentrations, semi-quantitative PCR method was used and comparisons were done by using Gene Tools software (Syngene, Cambridge, UK), employing intensities of the amplified bands separated on 1% agarose gel. This software can quantify the fluorescence intensity from electrophoresis bands and enables the investigation of gene expression levels [29].

To determine the flavonoid content, 0.1 g of fresh sample was ground into powder in 10 ml ethyl alcohol: acetic acid (1:99, v/v) and centrifuged for 10 min at 4000 rpm. The supernatant was slowly heated over a hot water bath for 10 min at 80 °C. Absorbance measurement was done at wavelengths of 270, 300 and 330 nm, considering the extinction coefficient of 33,000 $M^{-1} \cdot cm^{-1}$. Flavonoid contents were calculated cumulatively and reported in $\mu M \cdot g^{-1}$ fresh weight (fw) [30].

To measure anthocyanin content, method of Krizek et al. (1993) was applied. Briefly, 0.2 g of fresh sample was ground into powder in 3 mL acidified (0.1% HCl) methanol and then centrifuged at 12000 rpm for 20 min at 4 °C. Supernatant as a pigment container was stored in darkness for 24 hours. Absorbance was measured at 550 nm and the extinction coefficient of 33,000 $M^{-1} \cdot cm^{-1}$

was used to calculate anthocyanin content. Finally, anthocyanin content was reported in $\mu M \cdot g^{-1}$ fw [31].

Protein was extracted through grounding 0.5 g of fresh sample into powder in 50 mM potassium phosphate buffer (pH 7.5) comprising 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The mixture was centrifuged at 11000 rpm for 20 min at 4 °C. The supernatant was used for measuring protein content and investigating enzyme activities [32]. Total content of protein was determined from plant extractions according to the Bradford's (1976) method by using Bovine serum albumin (BSA) as the standard and the absorbance was measured at 595 nm [33].

To measure superoxide dismutase (SOD; EC 1.15. 1.1) activity, method of Giannopolitis and Reis (1977) was used, which emphasizes on preventing photochemical reduction of nitro blue tetrazolium (NBT) through the enzyme's capability. One unit of the enzyme activity is defined as the amount of enzyme required to 50% inhibition of NBT reduction into blue formazan under light conditions. Reaction mixture included 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 50 mM potassium phosphate buffer (pH 7.8) and 0.1 mM EDTA. Finally, absorbance was measured at 650 nm [34].

To measure catalase (CAT: EC 1.11. 1.6) activity, method of Dhinsda et al. (1981) was used, which relies on enzyme's capability to degrade H_2O_2 in 1 min. One unit of catalase enzyme is defined as the amount of enzyme, which degrades 1 ml of H_2O_2 per minute. The reaction mixture included 15 mM H_2O_2 and 50 mM potassium phosphate buffer (pH 7). Amount of H_2O_2 in reaction mixture after 1 min and absorption differences were measured at 240 nm and then the activity was defined in $U \cdot mg^{-1}$ protein [35].

To assay peroxidase (POD; EC 1.11. 1.7) activity, method of Plewa et al. (1991) was applied, which concentrates on absorbance of tetraguaiacol formed by oxidation of guaiacol which, was catalyzed by peroxidase at 470 nm in 3 min. Reaction mixture contained 4% guaiacol, 1% H_2O_2 and 50 mM potassium phosphate buffer (pH 7). POD activity was measured using an extinction coefficient of tetraguaiacol, $\varepsilon = 26.6\ mM^{-1} \cdot cm^{-1}$ [36].

All experiments were done through completely random designs with 3 independent replicates. Data means were used for Duncan's multiple range test. Afterwards, one-way analysis of variance (ANOVA) with a significance level of 0.05 was used for analyses of data with SAS 9.1.3 (SAS Institute, Cary, NC).

Results. Treatment of lemon balm seedlings with different Cu^{2+} concentrations led to different responses of RA production. RA content of experimental samples, compared to the standard sample, was measured after 16 minutes retention time (data not shown). The maximum amount of RA reached about 36 mg/g dry weight, in treatment with 5 μM Cu^{2+} after 8 hours, which was 3.5 fold more than the control (8.5 mg/g) (Fig. 1, A). Treatment with 10 μM Cu^{2+} resulted in 12 mg/g RA, being significantly bigger than the control. RA content of samples treated with higher Cu^{2+} concentration was similar to the control. The RA concentration was reduced after 16 hours of treatment and only 10 μM Cu^{2+} treatment was comparable to the results at 8 hour timepoint.

TAT gene expression was studied in a semi-quantitative method. Extracted RNA quality was determined by 18S and 28S bands related to ribosomal RNA on 1% agarose gel, which were clearly observable for all samples, indicating high quality of extracted RNA (Fig. 2, A). After cDNA library construction and TAT gene amplification, the gene expression level was calculated through loading of 4 μl of each PCR product (242 bp in length) on 1% agarose gel by using Gene Tools software (Fig. 2, B).

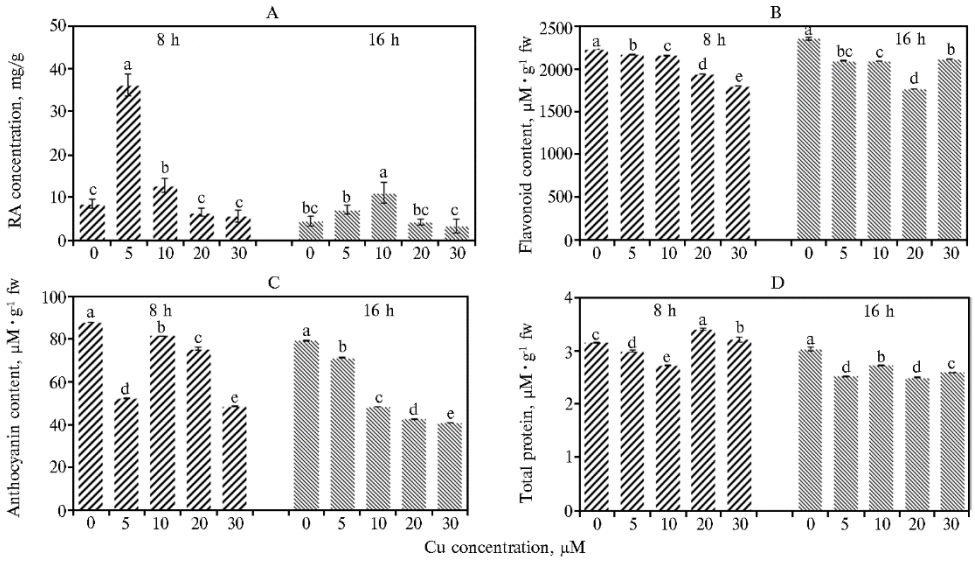

Fig. 1. Rosmarinic acid (RA) (A)**, flavonoids** (B)**, anthocyanin** (C)**, and protein** (D) **content of lemon balm treated seedlings with different Cu^{2+} concentrations after 8 and 16 hours (***n* = 3, *M*±SD, lab tests). Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test.

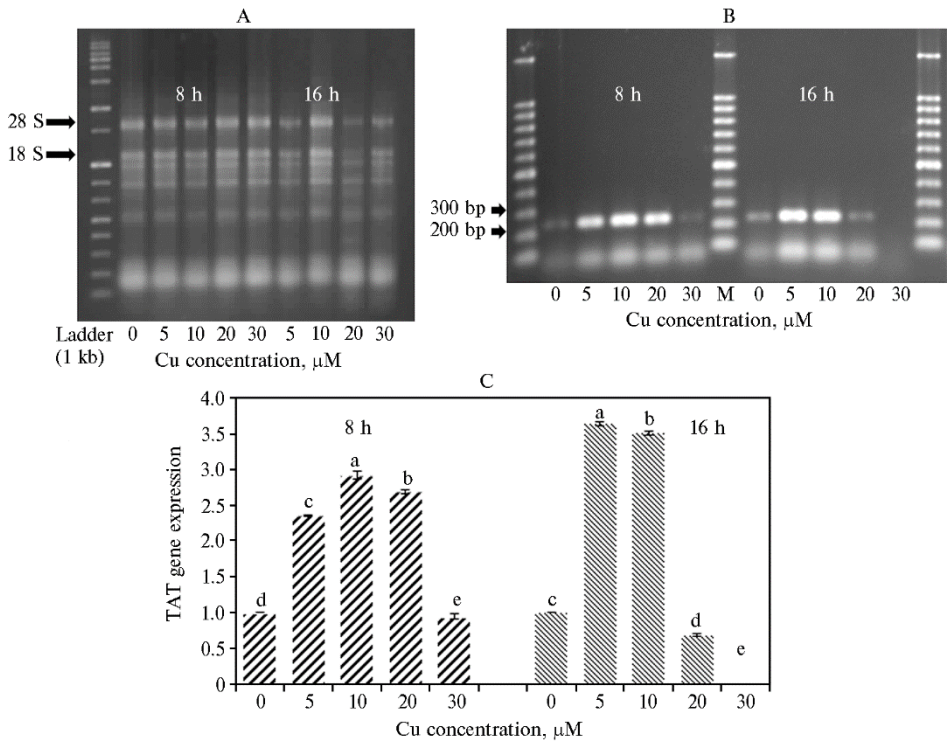

Fig. 2. RNA extracted from Cu^{2+}-treated lemon balm seedlings (A)**, amplified TAT gene on agarose gel** (B)**, and comparing of TAT gene expression** (C) **in treated seedlings with different concentrations of Cu^{2+} after 8 and 16 hours** (*n* = 3, *M*±SD, lab tests). GelPilot 1 kb Ladder (Qiagen, Germany)**.** Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test.

Treatment of seedlings with 5, 10 and 20 μM concentrations of Cu^{2+} for 8 hours led to an increase in TAT gene expression level compered to control (Fig. 2, C). Similarly, the seedlings treated with 5 and 10 μM of Cu^{2+} for 16 hours showed a significant rise in the level of the expression. On the other hand, by

increasing Cu^{2+} concentration in media, expression of this gene was decreased which was more considerable in 16 hours timepoint, while no band was observed on the gel in 30 μM concentration of Cu^{2+}.

Flavonoid content of the Cu^{2+}-treated seedlings tended to decrease with the increase of ions concentration in both treatment times (Fig. 1, B). Decrease in the flavonoid content was comparatively bigger at 16 hours than 8 hours although 30 μM Cu^{2+} concentration treatment recovered flavonoid content approximately up to level of 5 μM Cu^{2+}.

Treatment of lemon balm seedlings with different Cu^{2+} concentrations at the both treatment times led to a tendency to decrease in anthocyanin content compared with control (Fig. 1, C). Decrease in anthocyanin content was more considerable at 16 hours than 8 hours as well as in higher Cu^{2+} concentrations.

Treatment of lemon balm seedlings with different Cu^{2+} concentrations at the both treatment times led to significant increase in the SOD activity and to significant decrease in CAT activity compared to control (Table).

SOD, CAT and POD activity of treated lemon balm seedlings with various concentrations of Cu^{2+} at different treatment times (n = 3, M±SD, lab tests)

Cu^{2+} concentration, μM	SOD, U/mg protein		CAT, U/mg protein		POD, U/mg protein	
	8 h	16 h	8 h	16 h	8 h	16 h
0	38.4±7.7^{c}	51.6±17.2^{e}	179.7±2.3^{a}	161.9±1.8^{a}	3.61±0.4^{b}	3.51±0.3^{d}
5	395.6±15.6^{a}	142.8±10.8^{d}	89.3±1.1^{d}	156.7±0.9^{b}	6.29±0.5^{a}	7.65±0.4^{b}
10	277.0±19.7^{b}	232.4±21.7^{c}	157.0±1.1^{b}	105.7±1.3^{c}	5.87±0.1^{a}	6.15±0.2^{c}
20	376.8±16.3^{a}	382.9±25.5^{a}	86.6±1.1^{e}	93.7±1.8^{d}	5.58±0.4^{a}	8.86±0.4^{a}
30	397.9±14.5^{a}	301.5±17.1^{b}	107.3±0.7^{c}	65.9±1.4^{e}	5.81±0.1^{a}	6.99±0.4^{b}

N o t e. Different letters in each group indicate significance at $p \leq 0.05$ according to Duncan's Multiple Range Test.

POD activity was significantly increased in Cu^{2+}-treated seedlings proportionally to Cu^{2+} concentration increase in medium, except for 30 μM Cu^{2+} for 16-hour treatment, where the enzyme activity was the same as the level of 5 μM Cu^{2+} (see Table).

Soluble protein content of the Cu^{2+}-treated seedlings significantly decreased in 5 and 10 μM treatments at both timepoints. At 8-hour treatment in presence of 20 and 30 μM Cu the protein content increased instead (Fig. 1, D). However, longer treatment still significantly decreased the protein content compared to the control.

In the present study, effects of different Cu^{2+} concentrations were analyzed on RA accumulation, TAT gene expression as well as antioxidant system in 45-day-old *M. officinalis* seedlings at different timepoints. According to the results, copper had positive effect on the RA content especially at lower doses (5 and 10 μM) whereas at higher concentrations RA content was similar to the level of control. The maximum amount of RA was obtained in the presence of 5 μM Cu^{2+} after 8 hours of treatment, when RA concentration increased 3.5 times more than the control. These observations were consistent with the TAT gene expression profiles. On the other hand, although the maximum gene expression was seen in the presence of 5 and 10 μM Cu^{2+} after 16 hours of treatment, it was completely suppressed at 30 μM Cu^{2+}. Interestingly, in comparison with control, flavonoid and anthocyanin contents significantly decreased when the seedlings were treated with the different concentrations of this ion at the treatment timepoints, particularly at 16-hour treatment. Since flavonoid, anthocyanin and RA are phenolic compounds, it may be suggested that RA is produced more through tyrosine derived pathway [37]. Furthermore, as recently reported by Nasiri-Bezenjani et al. (2014), RA and TAT gene expression of lemon balm seedlings were induced by yeast extract after 17 hours and the data revealed these two parameters had a

similar pattern. Decrease in flavonoid and anthocyanin content was also consistent with our recent report, which showed the decrease of these metabolites when lemon balm seedlings were treated with Fe^{2+} concentrations [27]. Treatment of *Pinus sylvestris* L. seedlings with high levels of copper or nickel showed a decline in their phenolic compounds contents [38]. Based on this report, the decrease of phenolic compounds content possibly is related to suppression of other genes such as PAL, which are involved in their biosynthesis pathway or degradation of the enzymes involved in this pathway due to high toxicity of these ions in the medium. PAL, one of the most important enzymes in phenylpropanoids pathway [1], can be activated under stress conditions in plants to protect the plant during the synthesis of secondary metabolites such as simple phenols, anthocyanin, flavonoids, and lignins [39]. Previous studies have shown an increase of PAL activity in the *Phyllanthus tenellus* and *Camellia sinensis* leaves treated with copper, mercury and nickel [14, 15].

In comparison with other abiotic stresses, heavy metals induce the synthesis of heat shock proteins (HSPs), messenger molecules such as salicylic acid, abscisic acid and jasmonates and ethylene [40]. It has been proposed that free radicals have reciprocal roles in cells, while at higher concentrations they may damage cell membrane, nucleic acids and proteins [41], at lower concentrations they can act as signalling molecules [40]. Jasmonate, as a key signalling molecule, plays an important role in signalling network adjustment, which leads to biosynthesis of plant secondary metabolites [42]. The effects of these compounds on biosynthesis of different types of secondary metabolites such as alkaloids, terpenoids, glycosinolates and phenylpropanoids have been clarified [43]. Rapid accumulation of jasmonic acid in plant species such as *Phaseolus coccineus* and *Arabidopsis thaliana* after treatment with copper was proved [44]. Increase in RA production in exposure to some elicitors (yeast extract and methyl jasmonate) in several plant species including *Orthosiphon aristatus* [45], *Coleus blumei* [46] and *M. officinalis* [28] were reported. Yan et al. (2006) declared that increase of RA content in *Salvia miltiorrhiza* after treatment with yeast extract and silver ion is due to an accretion in activity of TAT gene. By considering the antioxidant properties of RA [47], it can be proposed that increase in RA content in Cu^{2+}-treated seedlings may be attributed to the induction of ROS and activation of a signaling pathway such as internal jasmonate [48], and consequently, activation of the genes involved in defensive system (e.g. TAT). Decrease in the level of these compounds due to antioxidant effects is related to the activation of other antioxidant pathways such as enzymatic pathway or degradation and inactivation of the enzymes, which are involved in biosynthetic pathways of these compounds [49]. Interestingly, SOD, which is the first enzyme in detoxification of ROS [50] and plays an important role in conversion of superoxide radicals to H_2O_2 and O_2 [51], increased in Cu^{2+}-treated seedlings compared with control after 8-hours exposure. In other words, in Cu^{2+}-treated seedlings, concentration of POD, which participates in removal of hydrogen peroxide inside the cell [52], was significantly increased in this research. However, the activity of CAT, another H_2O_2 scavenging enzyme in peroxisomes [53], was decreased significantly in Cu^{2+}-treated seedlings. Thus, it seems that lemon balm seedlings remove free radicals using expression of antioxidant enzymes such as SOD and POD.

Many studies showed that SOD, CAT and POD are necessary for protection of cells against side effects of ROS [50]. Miteva et al. (2005) declared that addition of arsenic acid concentration to culture medium led to significant increase in POD activity in tomato *(Lycopersicon esculentum* Mill.), which indicates

confrontation of POD with harmful effects of oxidative stress [54]. Moreover, significant increases in activities of some enzymes such as peroxidase and superoxide dismutase were reported in red cabbage (*Brassica oleracea*). Aforementioned plant used enzymatic and non-enzymatic antioxidants during heavy metal stress [55]. Wang et al. (2004) demonstrated that treatment of *Brassica Junica* seedlings with copper led to increase in the activities of SOD, POD, and decrease in CAT activity [56] which is consistent to our current study. Decrease in CAT activity may be due to lower affinity to combine with H_2O_2 or inhibition due to presence of high concentrations of heavy metals such as copper [57, 58]. It is pertinent to mention that cadmium caused CAT oxidation in pea (*Pisum sativum* L.) and as a result leading to a decrease in its activity [44]. Additionally, under toxic conditions of heavy metals, lack of a system to neutralize the by-products of oxygen radicals resulted in production of hydroxyl radicals through Fenton and Haber-Weiss reactions [59]. These radicals can change enzymatic activities, gene expression, protein content and soluble sugar content as well as release calcium from cell spaces, and also they can cause irreversible damages to plasma membrane and nucleic acid as were reported earlier [60]. Hence, decrease in protein content in Cu^{2+}-treated seedlings can be attributed to accumulation of these radicals. Decrease in protein content in wheat seedlings in presence of Cu^{2+} was shown by Singh et al. (2007) [61]. Furthermore, Singh et al. (2006) reported that protein content of *Pteris vittata* was decreased due to oxidative stress resulted from arsenic acid and degradation of some proteins [62]. On the other hand, elevated levels of soluble proteins at 20 and 30 μM after 8 hours of treatment may be attributed to the synthesis of heat shock proteins. Thereby, decrease in total flavonoid content, anthocyanin and RA in Cu^{2+}-treated seedlings may be attributed to inhibition of the enzymes, which are involved in RA biosynthesis pathway or may be attributed to degradation of these enzymes due to oxidative stress.

It can be concluded that the Cu^{2+} ion at investigated concentrations in this study leads to oxidative stress due to inducing the production of free radicals. Thus, production of these radicals, specially hydrogen peroxide, directly activate signalling pathways or induce expression of other genes involved in the pathway through biosynthesis of certain compounds such as plant hormones [48]. Increase in TAT gene expression level can be due to signalling roles of these radicals, which in turn leads to synthesis of RA when the seedling are treated with low concentrations of Cu^{2+}. Although the production of free radicals usually coincides with increase of SOD and POD activities, higher concentration of free radicals leads to degradation of some proteins or their functions or decrease in gene expression of these proteins. This assumption is consistent with the observed decrease in total protein content as well as activities of antioxidant enzymes and severe decrease in TAT gene expression in the treatment with highest copper concentration. This issue can also be due to decrease in flavonoid and anthocyanin content along with decrease of RA production in Cu^{2+}-treated seedlings at higher ion concentrations. Future experiments could test the effect of copper on RA in soil systems. If copper can increase RA content, it can be used in crop production to enhance the valorization properties of lemon balm and to help bioeconomy.

To summarize, lower concentrations of Cu^{2+} caused oxidative stress, followed by accumulation of ROS as signals molecules, which induced RA accumulation by an increase of TAT gene expression level. On contrary, at the highest applied Cu^{2+} concentration for 16 hours, ROS suppressed TAT gene expression and decreased RA production, where elevated levels of superoxide dismutase and peroxidase activities were measured in these seedlings.

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EXTRACTION AND PHYSICOCHEMICAL CHARACTERIZATION OF PECTIN POLYSACCHARIDES FROM AMARANTH LEAVES

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Abstract

Polysaccharides are one of the most important classes of natural compounds that have practical application in various fields of science and technology. Pectin remains one of the most essential polysaccharides, being a primary constituent of the structural elements of the cell wall in higher plants, performs the functions of binding and strengthening components of the cell wall, and also regulates water metabolism. Pectic substances are widely used in medicine as detoxicants of heavy metals and regulators of metabolic processes in the human body. In addition, they are also a universal food additive (E440). Despite significant amounts of traditional raw material resources (apple and citrus pomace, beet pulp), new alternative sources of raw materials are being searched for, including vegetable plants introduced in Russia for the production of pectins and their use in the production of functional foods. Among non-traditional plant resources, amaranth (*Amaranthaceae*) holds a significant rank. Due to its high yield and high content of biologically active substances and antioxidants, this crop acts as a potential source of obtaining valuable plant-derived substances for medicine, agriculture and the food industry. The *Amaranthus tricolor* L. cv. Valentina plants were used to isolate pectic substances by the classical method and ultrasonic treatment at a frequency of 22 kHz. Sugars were quantified using a Shimadzu 20-AD Prominence liquid chromatograph (Shimadzu Corporation, Japan) with a Shimadzu RID-10A refractometric detector. Infrared spectra were recorded on an IRS-113 instrument (Bruker, Germany) with a resolution of 1 cm^{-1} in the range 400-4000 cm^{-1}. The elemental composition was determined (a CHNSO-high-temperature analyzer Euro EA 3028-HT-OM, EuroVector Instruments & Software, Italy). All measurements by atomic force microscopy (AFM) were carried out on a Multi Mode V scanning probe microscope (Veeco Instruments, Inc., USA) in an intermittent contact mode. Structural study of the isolated polysaccharides by the IR spectroscopy method showed their possible affiliation to pectin substances. To study the monosaccharide composition, the samples of pectins were hydrolyzed with sulfuric acid (2 N) and partially hydrolyzed with trifluoroacetic acid (TFA). The high-performance liquid chromatography (HPLC) identified glucose, galactose, rhamnose, arabinose and galacturonic acid in the pectin fractions. Low galacturonic acid contents of 0.63 % and 1.68 % were determined in H_2SO_4 and TFA hydrolyzates, respectively. The conditions for hydrolysis-extraction which ensure the maximum yield of pectin substances were 0.5 % oxalic acid with complexone (0.5 % HDTA), 50-55 °C, 4 h, feed to extractant volume (hydromodule) ratio of 1:15. The physicochemical properties of pectin obtained under these conditions were studied using atomic force microscopy (AFM) and thermogravimetric/differential scanning calorimetry (TG/DSC) methods. After ethanol re-precipitation, this pectin sample showed an intense absorption band of stretching vibrations of carbonyls of carboxyl groups and ester groups at 1742 cm^{-1}. The TG/DSC indicated a two-step weight loss. The Fourier-transform infrared (FTIR) spectrum of the gaseous products derived from thermal decomposition of pectin sample showed that water was the main component of the gas phase at the first stage

of weight loss and at the second stage, pectin was decarboxylated. According to atomic force microscopy, the size of the aggregates was 2.4-2.5 μm maximum and ~ 330 nm minimum.

Keywords: *Amaranthus tricolor* L., amaranth, cv. Valentina, hydrolysis-extraction, ultrasonic disintegrator, pectin, IR spectroscopy, pectin thermostability, TG/DSC, AFM

Polysaccharides are one of the most important classes of natural compounds that have practical applications in various fields of science and technology. Pectin, a special polysaccharide of *Amaranthus* L. is a structural element of the cell tissue of higher plants, acts as a binding and strengthening component of the cell wall, and regulates water metabolism [1]. Pectins are widely used in medicine as heavy metal detoxifiers and metabolic regulators in humans, and also serve as a universal food additive (E 440) [2]. Despite the significant volumes of traditional raw materials (apple, citrus, and beet pulp), there is a search for new non-traditional raw materials for the pectins to be used in the functional food production [3-6]. Amaranth plants due to high yield and high content of biologically active substances and antioxidants is a potential source of valuable substances for medicine, agriculture and food industry [7-9]. The bioactive peptides derived from amaranth proteins exhibit antimicrobial, antioxidant, and antihypertensive activity [10-12], hypocholesterolemic effect [13] and antitumor activity [14].

Screening of the chemical composition of amaranth plants of various species showed that its biomass (leaves, inflorescences, stems) contains a number of chemical compounds of practical value. These are waxes, carotenoids, flavonoids, proteins, polysaccharides and other substances. Determination of their chemical composition and development of isolation methods open up prospects for obtaining functional products [6, 16, 17].

In this work, for the first time, the influence of the hydrolyzing agent and ultrasonic treatment (22 kHz) on the efficiency of hydrolysis, extraction, and the yield of pectin during isolation from amaranth variety Valentina plants is shown compared to the classical method.

Our goal was to develop methods for extracting pectin polysaccharides from *Amaranthus tricolor* L. cv. Valentina plants and to characterize the physicochemical properties and structural features of the extracted compounds.

Materials and methods. Vegetable amaranth cv. Valentina (bred at the All-Russian Research Institute of Vegetable Breeding and Seed Production VNIISSOK; included in the State Register and approved for industrial use) [18, 19] were grown without the use of pesticides and herbicides (the VNIISSOK experimental fields, Moscow Province).

In experiment 1, a flat-bottomed flask with dry ground *A. tricolor* leaves (100 g) were filled with distilled water and allowed at room temperature to isolate the amaranthine pigments. The dried depigmented raw material (85 g) was poured with 0.5% oxalic acid and added with a complexone (0.5% hexamethylenediaminotetraacetic acid, HDTC) to bind divalent metal ions. After 4-hour extraction (a hydromodulus of 1:15, pH 3.8, 50-55 °C) the extract was separated by filtration, evaporated to 200 ml at 60 °C under vacuum and precipitated with acetone (1:1 v/v). The precipitates were dried (a SNOL 58/350 laboratory oven, JSC Umega, Lithuania) at 40-50 °C to form films. The mass of dried pectin polysaccharide (sample 1) was 2.980 g.

In experiment 2, a flat-bottomed flask with dry ground raw materials (165 g) was filled with distilled water. Portions of dried depigmented raw material (108 g) was added with 0.5% citric acid and extracted without a complexone for 5 hours (a hydromodule of 1:14, pH 3.8, 50-55 °C). The extract was separated using a fabric filter, evaporated to 200 ml at 60 °C under vacuum and precipitated with acetone (1:1.5 v/v). The precipitate was dried at 40-50 °C; the weight of the

dried precipitate (sample 2) was 0.570 g.

To intensify hydrolysis and extraction, in two other experiments, ultrasonic treatment was applied (a disintegrator UZDN-1, Russia). Amaranthine was preliminarily removed with water from ground amaranth leaves (100 g). The resulting depigmented raw material was divided into two parts for extractions with 1.0% oxalic or 1.0% citric acid.

In experiment 3, pectin substances were extracted from raw materials (32 g) with 1.0% oxalic acid during ultrasonic treatment (UZDN-1, 22 kHz) for 5 min (a hydromodule of 1:10) with heating (11-37 °C). The extract was filtered and centrifuged (a centrifuge Sigma 4-15, Sigma-Aldrich, Germany). The product was precipitated with an equal volume of acetone and dried at 60 °C. The weight of the dried precipitate (sample 3) is 0.420 g.

In experiment 4, ground raw materials (32 g) were extracted with 1.0% citric acid under sonication (UZDN-1, 22 kHz) for 5 min with heating (23-45 °C, a hydromodule of 1:10). The extract separated from the pulp was added with an equal volume of acetone to precipitate pectin polysaccharides. The weight of the dried product (sample 4) is 0.560 g.

The isolated pectin substances were purified by re-precipitation with pretreatment with an ion-exchange resin (KU-2 cation exchanger) for demineralization. The concentration of pectin in the extracts was measured by the calcium pectate method.

Partial hydrolysis was performed to identify sugars in the side links of pectin polymers. A portion of the sample was hydrolyzed with trifluoroacetic acid (TFA) at 120 °C for 1 hour. TFA was distilled off under vacuum. To determine the monosaccharide composition, a sample of pectin was hydrolyzed with 2 N sulfuric acid at 110 °C for 5 hours. The hydrolyzate was neutralized with barium hydroxide, the precipitate was separated by filtration. The filtrates were analyzed by paper chromatography (ZMM chromatographic paper, Czech Republic; n-butanol:acetic acid:water solvent system, 5:1:4; aniline hydrogen phthalate as a reagent for developing chromatograms). Silufol UV-254 plates (Kavalier, Czech Republic) were used for thin-layer chromatography (n-butanol:acetic acid:water solvent system, 5:4:1; aniline hydrogen phthalate as a developer); monosaccharides (Sigma-Aldrich, Germany) were used as markers.

The sugars were quantified using a Shimadzu 20-AD Prominence liquid chromatograph (Shimadzu Corporation, Japan) with a Shimadzu RID-10A refractometric detector and ReproSil-Pur NH_2 (250 × 4 mm) (Dr. Masch GmbH, Germany) and YMC-Pack Polyamine II (250 × 4.6 mm) (YMC Europe GmbH, Germany) chromatographic columns. The acetonitrile:water 75:25 (v/v) eluent was the mobile phase (the eluent flow rate 1 ml/min). Calibration was carried out using standard solutions of monosaccharides (Sigma, Germany).

Infrared spectra (an infrared spectrographn IRS-113, Bruker, Germany) were recorded with a 1 cm^{-1} resolution in the range of 400-4000 cm^{-1}in KBr pellets. The elemental composition was determined (an elemental CHNSO-high-temperature analyzer Euro EA 3028-HT-OM, EuroVector Instruments & Software, Italy) with streptocide as an analytical standard (C — 41.85%, H — 4.65%, N — 16.26%, and S — 18.62%).

All measurements by atomic force microscopy (AFM) were performed on a Multi Mode V scanning probe microscope (Veeco Instruments, Inc., USA) in the intermittent-contact mode. Scanning was performed using RTESP rectangular cantilevers (Veeco Instruments, Inc., USA) with silicone probes. The resonant frequency of the cantilevers ranged was 250-350 kHz, the probe curvature radius was 10-13 nm. Microscopic image resolution was 512×512 points per

frame at a scanning speed of 1 Hz. To eliminate the distortions associated with the external noise-caused "trembling" of the microscope, an anti-vibration system capable of smoothing oscillations up to 0.5 Hz (lower limit) (TMC Vibration control, USA) was used. IR samples were prepared by applying 7 μl of a pectin solution onto a mica substrate, followed by deposition of pectin particles on it.

Ultrafiltration was carried out using an automatic ultrafiltration unit AUF-0.6 (TOO Vzor, Russia) with hollow fibers. The filtration limit is 50 kDa, the operating pressure during filtration is 0.8-1.2 MPa, and the filtering surface area is 200 cm2.

To study the thermal decomposition of amaranth pectin, we used the method for synchronous thermal analysis — thermogravimetry/differential scanning calorimetry with IR-Fourier spectroscopy (TG/DSC) was used which records the change in the mass of the sample under thermal effects vs. temperature. A coupled system consisting of a TG/DSC STA449-F3 simultaneous thermal analysis instrument (Netzsch, Germany) and a Tensor 27 IR-Fourier spectrometer (Bruker, Germany) was used.

Results. In our experiments on the isolation of amaranthine from the leaves of amaranth variety Valentina by water extraction followed by extraction of pectin substances, oxalic and citric acids were hydrolyzing agents at the hydrolysis-and-extraction stage. The optimized technological parameters were temperature, pH, hydromodules, and time. We believed that ultrasonic treatment intensifies the procedures. Table 1 shows the extraction parameters and the yield of pectins.

The results showed that the UZDN-1 treatment significantly reduces the time of the extraction. In addition, when using citric acid, the weight of isolated pectin polysaccharides turned out to be higher than when extracting with oxalic acid (see Table 1). Perhaps the difference is due to the extraction conditions, namely, heating the sample with citric acid to 20 °C prior to ultrasonication.

1. Isolation of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants by acid hydrolysis

Hydrolyzing agent	Extraction mode				Pectin, g	Yield (per absolute dry weight), %
	time	T, °C	hydromodule	kHz		
0.5 % oxalic acid + 0.5 % HDTA	4 h	50-55	1:15	–	2.98	3.51
0.5 % citric acid	5 h	50-55	1:14	–	0.57	0.53
1.0 % oxalic acid	5 min	11-37	1:10	22	0.42	0.63
1.0 % citric acid	5 min	23-45	1:10	22	0.56	1.75

N o t e. Ultrasonication was performed using a UZDN-1 device (Russia). Dashes mean that processing was not carried out. HDTA — hexamethylenediamine tetraacetic acid.

Data of the elemental analysis of pectin substances from amaranth cv. Valentina (C — 41.87%, H — 6.56%) showed the absence of protein when extracted with 0.5% oxalic acid + 0.5% HDTA and a high protein content (N — 4.43%) when extracted with 0.5% citric acid. After ultrasonication, extraction with both 0.5% oxalic acid and 0.5% citric acid resulted in 2.0% nitrogen content.

To determine the monosaccharide composition of the isolated pectin, we applied hydrolysis with 2 N sulfuric acid and partial TFA-assisted hydrolysis. Thin-layer chromatography showed a significant proportion of arabinose and galactose polymers (arabinans and galactans or arabinogalactans), and traces of rhamnose. HPLC revealed glucose, galactose, rhamnose, arabinose and galacturonic acid (8.3:7.7:4.1:6.6:71.0) in the pectin fractions, which is consistent with the report of Sarkar et al. [20]. A low content of galacturonic acid was characteristic of both hydrolysates (0.63 and 1.68% for H_2SO_4 and TFA, respectively). When extracted with 0.5% oxalic acid + 0.5% HDTA, rhamnose was not detected. Similar results were obtained by paper chromatography.

Infrared spectroscopy (IRS) is one of the most common methods for studying plant polysaccharides [21] used both to monitor extracts and to quantify acidic and neutral sugars in a polysaccharide. Structural study of the isolated polysaccharides by IR showed their correspondence to pectin substances (Table 2). In the IR spectra, a wide intense band at 3000-3600 cm^{-1} appeared in all samples, which is characteristic of the stretching vibrations of hydroxyl groups. The characteristic shift indicated that this is a band of stretching vibrations of −OH groups with a hydrogen bond. It was also shown that in the IR spectra of pectin substances there were characteristic absorption bands of different intensity corresponding to the stretching vibrations of carbonyls of carboxyl groups (at 1700-1750 cm^{-1}) and ester groups (at 1730-1750 cm^{-1}).

At 1632-1647 cm^{-1}, absorption bands corresponded to stretching vibrations of dissociated carboxyl groups. The absorption bands at 1020-1100 cm^{-1} corresponded to the stretching vibrations of the pyranose rings, and at 1325 cm^{-1} to in-plane vibrations of the −CH groups (for IR spectra of pectin extracted from amaranth cv. Valentina plant by acidic hydrolysis, see http://www.agrobiology.ru).

2. Peaks (cm^{-1}) **of the main characteristic absorption bands in the infrared spectra of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis** (IR-Fourier spectrometer IRS-113, Bruker, Germany)

Sample 1	Sample 2	Sample 3	Sample 4	Main types of vibrations
3444	3445	3427	3412	ν(OH), ν(H_2O)
2925	2923	2924	2921	ν(CH)
1739	1738	–	–	ν(C=O)
1634	1632	1647	1647	ν(COO^-)
1401	1406	1402	1403	ν, δ(C−OH)
1382, 1228	1238	1325, 1224	1375, 1325, 1294	δ(CH)
1022-1101	1022-1104	1029	1029-1155	ν(C−O−C), ν(C−C), ν(C−O)

N o t e. For extraction procedure, see the Materials and Methods section. Dashes indicate the absence of corresponding peaks.

Extraction with 0.5% oxalic acid + 0.5% HDTA without sonication resulted in the highest degree of pectin esterification. Salt carboxyls predominated in pectin isolated with UZDN-1 treatment (the sample with oxalic acid also contained a minor amount of free carboxyl groups). In both samples, there was a band at 1325 cm^{-1} (more pronounced for citric acid application), corresponding to in-plane deformation vibrations of −CH groups. In all treatments, apart from polysaccharides, proteins were extracted, and absorption bands of amide groups appeared in the IR spectra.

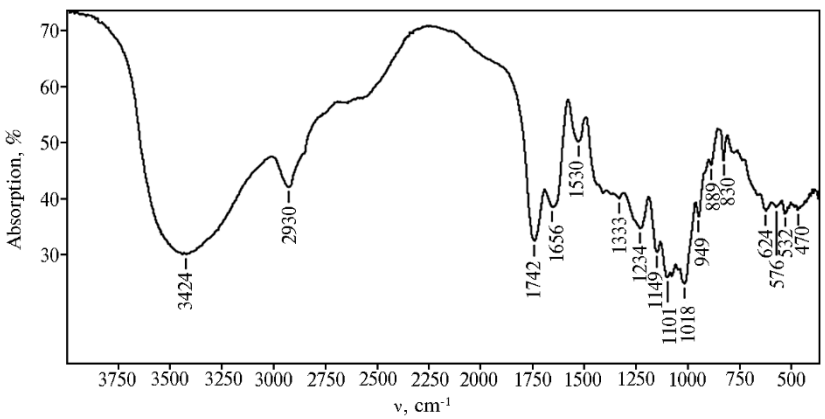

Fig. 1. Infrared spectrum of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis (0.5% oxalic acid solution + 0.5% hexamethylenediamine tetraacetic acid) and purified by re-precipitation with ethyl alcohol (IR-Fourier spectrometer Tensor 27, Bruker, Germany).

A combination of 0.5% oxalic acid + 0.5% HDTA as a hydrolyzing agent ensured the maximum pectin yield. Therefore, we additionally purified the pectin of *A. tricolor* cv. Valentina (sample 1) by re-precipitation with ethyl alcohol and characterized by IR spectroscopy, TG/DSC, and AFM. After re-precipitation, this sample had an intense absorption band of stretching vibrations of carbonyls of carboxyl groups and ester groups at 1742 cm^{-1} (Fig. 1).

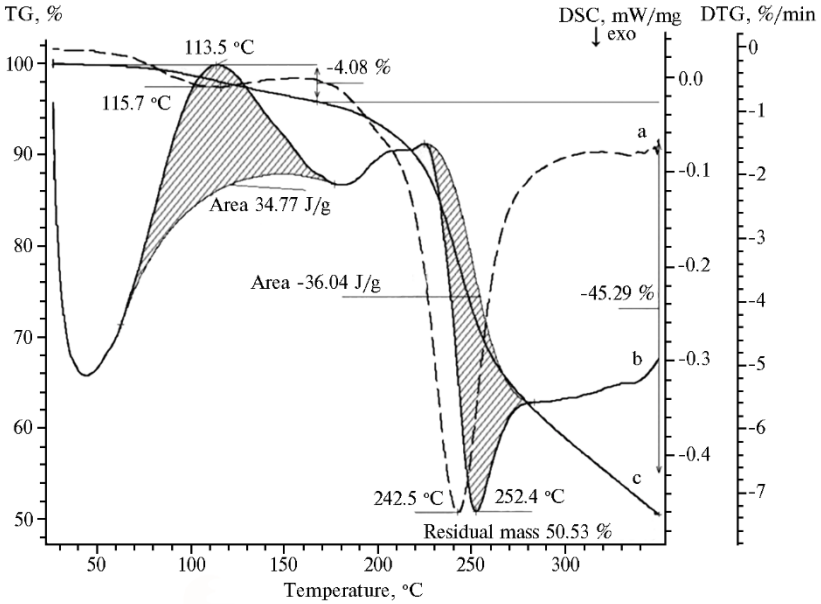

Fig. 2. Thermogravimetry/differential scanning calorimetry (TG/DSC) of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants extracted by acid hydrolysis (0.5% oxalic acid solution + 0.5% hexamethylenediamine tetraacetic acid) after re-precipitation with ethyl alcohol: a — Gram-Schmidt curve, b — DSC, c — TG. A STA449-F3 synchronous thermal analysis instrument (Netzsch, Germany) combined with a Tensor 27 IR-Fourier spectrometer (Bruker, Germany).

The thermal stability of pectin is one of the most important characteristics that determines the conditions for their storage and use in the confectionery industry. The TG/DSC data of pectin, which we isolated from amaranth cultivar Valentina (sample 1) and further purified by re-precipitation with ethyl alcohol, indicated a two-stage weight loss. The first stage had a peak at 117.3 °C with a weight loss on the TG curve of ~ 4.1% and was accompanied by an endothermic peak at 113.5 °C. Then, at 243.1 °C, the second stage of weight loss (~ 45.3%) occurred together with an exothermic peak with a ~ 36.04 J/g enthalpy (Fig. 2). The IR-Fourier spectrum of the gaseous products of the thermal decomposition of amaranth pectin showed that water was the main component of the gas phase at the first stage of weight loss; at the second stage, the decarboxylation of pectin occurred.

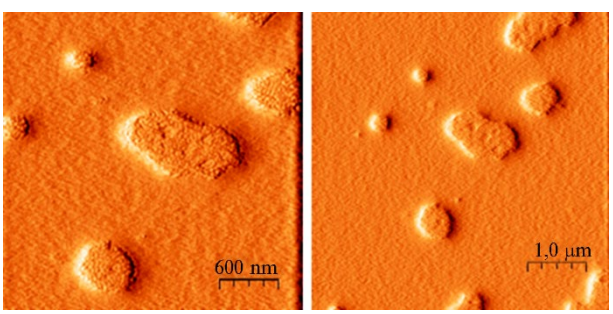

Fig. 3. Surface image of pectin from amaranth (*Amaranthus tricolor* L.) cv. Valentina plants obtained by acid hydrolysis (0.5% oxalic acid + 0.5% hexamethylenediamine tetraacetic acid) and re-precipitation with ethyl alcohol (atomic force microscopy method, scanning probe microscope Multi Mode V, Veeco Instruments Inc., USA).

AFM analysis showed that the maximum aggregate size in the pectin

sample 1 (concentration of solutions 10^{-4}%) was 2.4-2.5 μm, the minimum size was ~ 330 nm (Fig. 3). The aggregates mostly sized from 660 to 880 nm, the size of individual particles that made up the aggregates was 30-45 nm.

The results we obtained on the yield of *A. tricolor* cv. Valentina pectin per absolute dry mass and the physicochemical properties of the product are of practical interest for large-scale production for the food industry and pharmacology. The deterioration of the ecological situation and the increase in the number of various diseases necessitate detoxifying drugs and effective long-acting drugs based on pectin polysaccharides. It is known that amaranth pectin has a wide range of physiological activity [22, 23].

The traditional technology of pectin production widely uses the method of hydrolysis of protopectin with strong mineral acids. Currently, hydrochloric acid (HCl) is the main extracting solvent for the release of pectin from plant tissues. However, the use of mineral acids creates environmental problems [5, 9]. Green Chemistry provides for the development of fundamentally innovative approaches to reduce the anthropogenic load on the environment and avoid a harmful effect on humans. For environmentally friendly production, Cho et al. [24] extracted pectin from apple pomace with organic acids (tartaric, malic, and citric) at 85 °C. The obtained pectin was highly esterified and had high molecular weight (citric acid led to the maximum molecular weight). The yield of pectin extracted with organic acids was comparable to that during extraction with 0.1 M hydrochloric acid widely used f in industry. The results of these authors confirm the prospects and efficiency of using organic acids as a hydrolyzing agent and are consistent with our choice of oxalic and citric acids for obtaining pectin from *A. tricolor* cv. Valentina.

It should be noted that the extraction of pectin from non-traditional raw materials requires a thorough refinement of existing technologies, including not only the parameters of the main stages, but also searching for additional stages of processing raw materials and the resulting products to improve their quality. There is a technology based on the use of electro-activated water obtained by electrodialysis as a hydrolyzing agent. Rotary-pulsation-based and ultrasonication-based environmentally friendly technologies for pectin production are also reported, which intensify hydrolysis and extraction of pectin-containing raw materials and replace the traditionally used strong mineral acids with “soft” food acids [25]. In addition, there is a technology based on the use of microwave radiation [26]. Maran et al. [27] proposed the optimal conditions for microwave extraction (microwave power 413 W, pH 2.7, 134 s, hydromodule 1:18) providing the maximum pectin yield (28.86%).

Thus, the efficiency of hydrolysis and extraction of pectin substances depends on the hydrolyzing agent, pH, a hydromodule, time and temperature. We have proposed the protocol that provides the maximum yield (3.51%) of pectin from the *Amaranthus tricolor* L. cv. Valentina biomass extracted by the classical method: 0.5% oxalic acid + 0.5% hexamethyleneamine tetraacetic acid, 50-55 °C, 4 hours and hydromodule 1:15. Ultrasonic treatment at 22 kHz reduces the extraction time from 4 hours to 5 min, providing a pectin yield of 1.75%. Structural study of the extracted polysaccharides by infrared spectrometry showed their correspondence to pectin substances. High-performance liquid chromatography showed that the pectin fractions contained glucose, galactose, rhamnose, arabinose, and galacturonic acid monosaccharides. The content of galacturonic acid was 0.63% in the H_2SO_4 hydrolysate and 1.68% in the trifluoroacetic acid hydrolyzate. High molecular weight and degree of esterification allow us to recommend pectin from the amaranth cv. Valentina for use in the food industry.

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INFLUENCE OF META-CHLORO-BENZHYDRYL UREA ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *Saussurea orgaadayi* V. Khan. and Krasnob. CELL CULTURE

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Abstract

Meta-chloro-benzhydryl urea (m-CBU) is an inducer of the human monooxygenase system, its key enzymes belong to the cytochrome P-450 superfamily (CYP). Currently, there is no information about the role of m-CBU in the plant vital activity regulation; however, the participation of CYP in the metabolism of secondary metabolites, for example, flavonoids (Fl), and most phytohormones have been shown. *Saussurea orgaadayi* V. Khan. and Krasnob. is a poorly studied plant species. Its cell culture, in accordance with our data, doubles the total amount of endogenous Fl during the transition from exponential growth to the stationary phase. The present study, for the first time shows statistically significant ($p < 0.05$) differences in the growth responses of the callus culture of the *S. orgaadayi* to m-CBU in different concentrations. It was revealed that the change in the growth index in terms of fresh and dry weight is related to a change in the volume and shape of cells, as well as the occurrence frequency of different groups of cells. Here, for the first time, the dynamics of the content of Fl, accompanying changes in the growth of culture under the influence of m-CBU was assessed. The aim of this work was to determine the role of meta-chloro-benzhydryl urea in the accumulation of flavonoids and the variability of cytomorphological characteristics of *S. orgaadayi* callus culture (cell linear dimensions, volume and shape, the frequency of cells of different sizes, growth index for the fresh and dry biomass). A callus culture derived from cotyledon explants of sterile seedling was repeatedly passaged on a modified Murashige-Skoog (MS) agar nutrient medium supplemented with sucrose, vitamins and growth regulators 0.8 mg/l 2,4-D and 0.5 mg/l 6-BAP. The culture was grown in the dark at a temperature of 22-24 °C in MS medium added with growth regulators and 0.01, 0.1, 1, 10, or 100 μM m-CBU (Sintegal, LLC, Russia). In the control, m-CBU was not added. After 30 days of subculture, $^2/_3$ of the material was used to determine the wet and dry biomass followed by the isolation of flavonoids, and $^1/_3$ of the material was fixed in Clark's solution for 2 days. To prepare micropreparations, the cell culture was macerated in a 3 N hydrochloric acid solution with constant shaking until a homogeneous cell suspension was obtained. Cytophotometric analysis was performed using light microscopy (video camera Moticam 2300, Motic, Spain) with software. The sizes of 100 cells were measured for each variant, their shape was estimated, and the volume was calculated. To calculate the growth index (GI), the initial (beginning of subcultivation, M_0) and the final fresh or dry weight of calli (on day 30 of subculture, M_{30}) were determined and expressed as a percentage of the control: $GI = (M_{30} - M_0)/M_0$. The total amount of Fl in the callus culture was quantified based on the colored Fl complexation with aluminum chloride followed by measurement of the optical density (a UV-1650 spectrophotometer, Shimadzu Corp., Japan). As a result of the studies, a dose-dependent effect of m-CBU on cell growth was established due to their division (0.1 μM) and stretching (1-100 μM), which was accompanied by a 2.1-3.5-fold and 1.5-2.9-fold increase in the GI_f and GI_d of callus culture, respectively ($p < 0.05$). At a concentration of 0.1 μM, the number of small meristematic cells increased by 16 % compared to the control. At the same time, the average volume of large cells decreased by 31 % as compared to the control, which indicates inhibition of cell elongation processes. With the increase in m-CBU concentration, the frequency of cells of two groups increased, by 55 and 30 % for

medium-sized cells at 1 and 10 μM, respectively, and by 50 and 57 % for large-sized cells at 1 and 100 μM, respectively. The volume of cells also increased compared to the control, by 61 % at 10 μM for large cells and by 18 % at 100 μM of small cells. m-CBU reduced the total amount of endogenous flavonoids by 80-95 % ($p < 0.05$) upon activation of growth processes in *S. orgaadayi* cells in vitro. The content of the total Fl decreased maximally at 0.01 and 0.1 μM m-CBU and did not differ significantly from the control at 100 μM m-CBU. The maximum 3.5-fold increase in GI_f in the medium with 1 μM m-CBU occurred simultaneously with an 83 % decrease in the amount of Fl. m-CBU can be used in plant biotechnology as a cell growth modulator in callus cultures to reduce the content of growth-inhibiting metabolites. To activate cell division, the most preferable dose is 0.1 μM m-CBU, while to change the content of Fl, which doubles the biomass of the culture, 100 μM m-CBU should be used.

Keywords: *Saussurea orgaadayi*, cell culture, meta-chloro-benzhydryl urea, morphogenesis, flavonoids

Plants, unlike animals and humans, synthesize secondary metabolites, including flavonoids (Fls), the plant specialized metabolites of which more than 6900 compounds have been described [1]. These metabolites are synthesized from p-coumaroyl-CoA by the sequential action of various enzymes, which are believed to form ordered macromolecular multi-enzyme complexes of flavonoid enzymes weakly-bound to membranes (for example, to the endoplasmic reticulum), called flavonoid metabolons [1, 2]. Localization and duration of interactions between specific proteins play an important role in the synthesis of specific FLs during plant growth and response to stressors [1, 3]. FLs protect the plants against leaf-eating insects, pathogenic microorganisms, ultraviolet radiation and high-intensity light, attract pollinating insects, inhibit the formation of reactive oxygen species (ROS), participate in pollen germination, provide biological communication in the rhizosphere during nodulation, increase the efficiency of use nutrients during the aging of the plant and its organs [3-6]. They are capable of metal chelation, which may serve as an in vivo mechanism to reduce toxicity [3]. In addition, FLs act as developmental regulators involved in changes in the transport of the phytohormone indoleyl-3-acetic acid (IAA) and its metabolism [7].

Fls, not synthesized by animals and humans, are essential nutrients. Natural FLs are low toxic and serve for the prevention and treatment of various pathologies [8]. Dietary FLs with antioxidant activity reduce the incidence of atherosclerosis, cardiovascular diseases, diabetes, thrombosis, inflammation in arthritis, neurodegenerative diseases (Alzheimer's and Parkinson's diseases), obesity, hyperlipidemia, and hypertension [4]. Quercetin exerts an antiproliferative effect on cancer cell lines [9]. FLs are a raw material for the industrial production of pharmacological and cosmetic substances, which raises the question of controlling the content of FLs in plants.

Not only exogenous adverse biotic and abiotic factors but also endogenous factors, e.g., a hormonal balance associated with the age of the plant, its organs and cell cultures, can induce the FL biosynthesis [5, 10-12]. Cytochrome-P-450-dependent monooxygenases (CYP), the components of flavonoid metabolons are involved in the FL modification [1, 2, 13]. Meta-chlorobenzhydryl urea (m-CBU) is an inducer of the human cytochrome P-450-dependent monooxygenase system [14] but there is no information on the role of m-CBU in the regulation of plant vital activity.

Multiplicity of metabolic processes and structural elements in plants (at the cellular, tissue, and organ levels) hinders the study of molecular mechanisms. Thereof, heterotrophic cell cultures that are simpler in structure and do not exert photosynthetic activity can serve as the most successful models.

This paper shows for the first time statistically significant ($p \leq 0.05$) differences in the growth responses of the *Saussurea orgaadayi* V. Khan. and Krasnob. callus culture to various concentrations of m-CBU. It was found that the

growth rate (by fresh and dry weight) depends on the volume and shape of the cells and the proportion of different groups of cells. This is the first data on the dynamics of flavonoids as related to in vitro culture growth under the influence of m-CBU.

The work aimed to determine the role of meta-chlorobenzhydryl urea in the accumulation of flavonoids and the variability of cytomorphological parameters (linear dimensions, volume and shape of cells, the rate of different cell groups, the growth index for wet and dry callus biomass) of *S. orgaadayi* callus culture.

Materials and methods. Meta-chlorobenzhydryl urea (Galodif, CAS: 124057-07-4) was synthesized at the Tomsk Polytechnic University [15], the copyright holders of the drug are Syntegal LLC and Science, Technology, Medicine LLC (Russia). For 0.01, 0.1, 1, 10, and 100 μM m-CBU, a stock m-CBU alcohol solution (100 mM) was diluted.

Callus cultures induced from cotyledon explants of *S. orgaadayi* sterile seedlings were repeatedly subcultured on a modified agar Murashige-Skoog nutrient medium (MS) added with sucrose, vitamins and growth regulators 0.8 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.5 mg/l 6-BAP (6-benzynaminopurine) [16]. Calluses were grown in the dark at 22-24 °C on MS medium + growth regulators added with m-CBU (control was not added with m-CBU).

After 30-day subculture, $^2/_3$ of each callus was weighed to determine wet and dry mass and used for flavonoid isolation; $^1/_3$ portions were fixed in Clark's solution (96% ethyl alcohol:glacial acetic acid, 3:1) for 2 days, washed with 96% ethanol (3 times for 30 min) until the smell of acetic acid disappeared, and stored in 70% ethanol at 4 °C [17]. For micropreparations, the calli were macerated in 3 N hydrochloric acid solution with shaking until a homogeneous cell suspension is obtained.

Cytophotometric analysis was performed using light microscopy (Moticam 2300 video camera with software, Motic, Spain). The dimensions (L — length, D — width) of 100 cells were measured. Cell shape was assessed by the L/D ratio, the $1.0 \le L/D \le 1.14$ for round, $1.15 \le L/D \le 1.94$ for oval, and $L/D \ge 1.95$ for elongated cells. The cell volume was calculated according to the Tselniker's formula [18] with the correction factor (k) calculated by the author, which depends on L/D. At $L/D > 2.5$, to determine the cell volume (V, μm^3), the formula for the volume of a cylinder was used: $V = \pi(D/2)2Lk$. At $L/D \le 2.5$, the formula for a rotated ellipsoid was applied: $V = 4/3\pi L/2(D/2)^2$. To calculate the growth index (GI), the initial (at the beginning of subculture, M_0) and final wet or dry weight of calli on day 30 (M_{30}) were determined and expressed as a percentage of the control: $GI = (M_{30} - M_0)/M_0$.

Total FLs of *S. orgaadayi* callus culture were quantified by spectrophotometric assay based on a colored aluminium complex formation (a UV-1650 spectrophotometer, Shimadzu Corp., Japan) [19]. A portion of dry biomass (1 g) was extracted three times (for 60 min each) with 70% ethanol in a boiling water bath, and the extracts were combined. An aliquot of the bulk sample was added with aluminum chloride and acetic acid and allowed for 40 mi (a reference solution without aluminum chloride was prepared for each sample). The optical density of the test solution and the standard rutin solution was measured at $\lambda = 415$ nm. Total Fl content was expressed as rutin equivalents per absolute dry raw material (X_{Fl}): $X_{Fl} = OD_x \cdot K_x \cdot m_{rut} \cdot 100 \cdot 100 \cdot OD_{rut}^{-1} \cdot K_{rut}^{-1} \cdot m_x^{-1} \cdot (100 - W)^{-1}$, where OD_x is the optical density of the test solution; OD_{rut} is the optical density of the rutin solution; m_x is raw material mass, g; m_{rut} is the mass of rutin, g; K_x is the dilution factor of the test solution (1250); K_{rut} is dilution factor of a solution of rutin (2500); W is the weight loss from drying, %.

To perform statistical analysis (IBM SPSS Statistics for Windows, IBM Corporation, USA), the parametric Student's *t*-test and the nonparametric Mann-Whitney U-test for pairwise comparison of group parameters were used. The

figures show the arithmetic mean values (M) for growth (n = 100) and biochemical (n = 5) parameters with two-sided confidence intervals (M±1.96 SEM). Differences between values marked with different letters are statistically significant at $p < 0.05$.

Results. In our experiments, we studied morphogenesis and Fl accumulation in cotyledon-derived slowly growing callus culture of *S. orgaadayi* depending on the m-CBU concentration in the nutrient medium (Fig. 1). The choice of the crop was based on our preliminary studies [20] which showed a 3-fold excess of the Fl content (0.026±0.006% of dry weight) on day 25 in a slow-growing in vitro culture compared to actively growing culture from *S. orgaadayi* seedling hypocotyls.

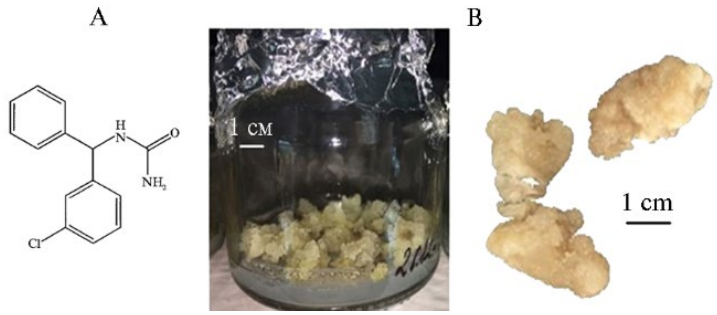

Fig. 1. Meta-chlorobenzhydryl urea (A) and callus cell culture derived from cotyledon explants of *Saussurea orgaadayi* V. Khan. and Krasnob. seedlings (B).

The time of callus subculturing followed from the pattern of an increase in wet weight of the control culture. The callus culture had an S-shaped growth curve showing several specific phases: a 10-day lag phase of low growth, a 15-day logarithmic phase, a 5-day deceleration phase, and a stationary phase starting from day 30 (data not shown). On day 25 of subculturing, the GI_{wet} of the callus in the control was 2.32±0.70. The experiment continued up to day 30 to reach the stationary phase and to increase the yield of secondary metabolites Fls, which is consistent with the data of other authors [12].

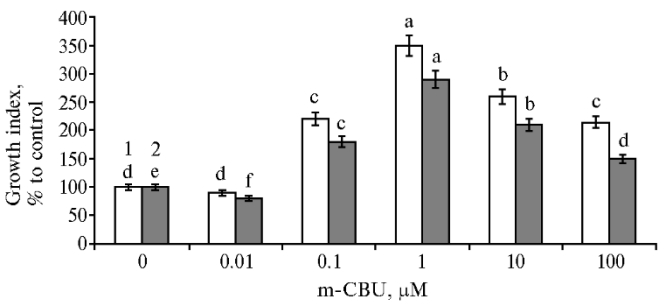

Fig. 2. Relative gain of raw (1) **and dry** (2) **weight of *Saussurea orgaadayi* V. Khan. and Krasnob. callus culture depending on the concentration of meta-chlorobenzhydryl urea** (m-CBU) **in the growth medium.** Differences between each indicator marked with different letters are statistically significant at $p < 0.05$ (M±1.96 SEM).

At 0.1-100 μM, m-CBU stimulated callus growth with a 2.1-3.5-fold increase in GI_{wet} and a 1.5-2.9-fold increase in GI_{dry} values ($p < 0.05$) as compared to the control (Fig. 2). At 0.01 μM, m-CBU did not change GI_{wet} but reduced GI_{dry} by 20% ($p < 0.05$). The GI_{wet} and GI_{dry} values were the highest at 1 μM m-CBU. The m-CBU-treated calli differed from the control in a lighter color and a looser consistency.

Cytological analysis revealed morphological heterogeneity of the *S. orgaadayi* cell population (Fig. 3, 4). We identified groups of cells that differed in shape (round, oval and elongated) and size (small, medium and large). We regarded small cells (9.0-35.7 thousand μm^3) as dividing meristematic cells, medium-sized cells (35.75-75.94 thousand μm^3) as growing, and large cells (76.0-323.2 thousand μm^3) as completed growth.

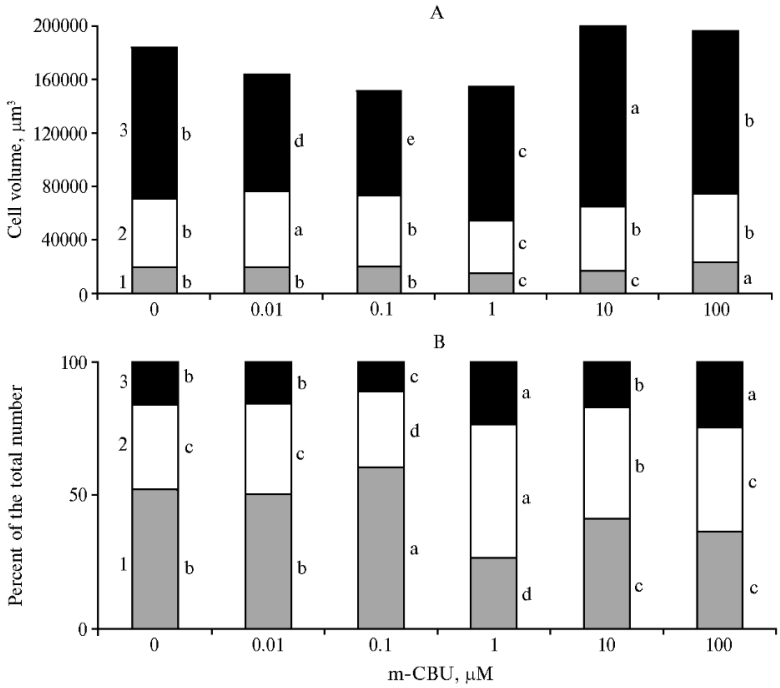

Fig. 3. Cell volume (A) **and the rate of small** (1)**, medium-sized** (2)**, and large** (3) **cells** (B) **in the *Saussurea orgaadayi* V. Khan. and Krasnob. callus culture depending on the concentration of meta-chlorobenzhydryl urea** (m-CBU) **in the growth medium**. Differences between each indicator marked with different letters are statistically significant at $p < 0.05$ ($M \pm 1.96$ SEM).

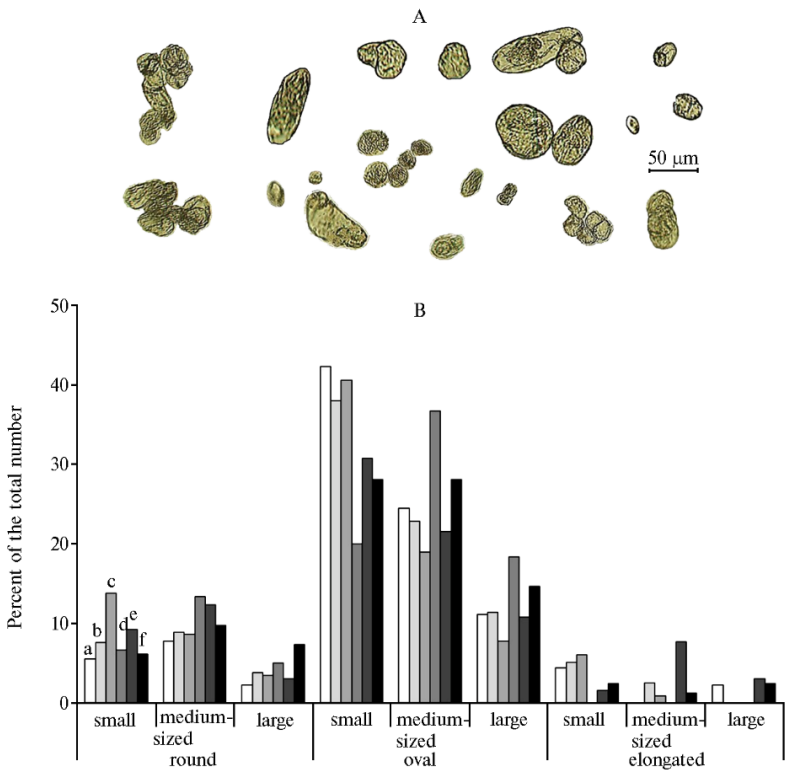

Fig. 4. Shape (A) **and the rate** (B) **of cells of various shapes and sizes in the *Saussurea orgaadayi* V. Khan. and Krasnob. callus culture depending on the concentration of meta-chlorobenzhydryl urea in the growth medium:** a — 0 (control); b — 0.01 μM; c — 0.1 μM; d — 1 μM; e — 10 μM; e — 100 μM.

In the control callus culture on day 30, the rate of small cells was 1.6 and 3.4 times higher than medium and large cells, respectively (Fig. 3, B). The average volume of small cells was 20.2±2.3 thousand μm^3, of medium and large cells 51.5±1.4 and 112.1±2.6 thousand μm^3 (Fig. 3, A).

The m-CBU was shown to affect cell growth in the calli. At 0.1 μM m-CBU, the number of small meristematic cells increased by 16% compared to the control, which could indicate activated cell division (see Fig. 3, B). The average volume of large cells decreased by 31% compared to the control (see Fig. 3, A), which indicated inhibition of cell elongation. With an increase in the m-CBU concentration, there was an increase in the rate of medium-sized cells by 55 and 30% for 1 and 10 μM and large cells by 50 and 57% ($p < 0.05$) for 1 and 100 μM (see Fig. 3, B). In large cells the volume increased by 61% ($p < 0.05$) for 10 μM, in small cells by 18% ($p < 0.05$) for 100 μM compared to the control. The number of small cells decreased by 49, 20, and 30% ($p < 0.05$) for 1, 10, and 100 μM m-CBU, and their volume decreased by 22 and 17% ($p < 0.05$) for 1 and 10 μM (see Fig. 3).

m-CBU increased the proportion of round cells of all sizes (Fig. 4, B). The greatest changes occurred in the rate of small cells (a 2.5-fofld increase, $p < 0.05$, for 0.1 μM m-CBU). The rate of medium-sized and large cells increased 1.7-fold and 2.3-fold, respectively ($p < 0.05$) for 1 μM m-CBU. The highest rate increased by 3.3 times ($p < 0.05$) was recorded for large round cells at 100 μM m-CBU.

A change in growth processes in the cell culture could indicate a change in the levels of endogenous substances that regulate growth. Among the latter, there are Fl modulating the homeostasis of plant hormones auxins [7]. We found out that the total content of Fl in the control subcultured calli at the stationary stage (day 30) was 0.049±0.008% of dry weight, which is 50% ($p < 0.05$) more than on day 25 at the inflection point of the logarithmic section of the growth curve. These data indicate a slowdown in growth with an increase in the of endogenous Fl content.

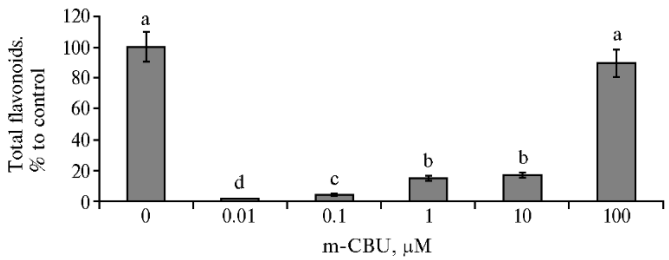

Fig. 5. Total flavonoids in the *Saussurea orgaadayi* V. Khan. and Krasnob. callus culture depending on the concentration of meta-chlorobenzhydryl urea (m-CBU) **in the growth medium.** Differences between each indicator marked with different letters are statistically significant at $p < 0.05$ (M±1.96 SEM).

Under the action of m-CBU, the total content of Fl changed compared to the control (Fig. 5). It was shown for the first time that a decrease (by 80-95%) in the total amount of endogenous Fl caused by m-CBU activates cell growth in the *S. orgaadayi* in vitro cell culture. The total Fl content decreased to its minimum at low m-CBU concentrations and remained at the control level at 100 μM m-CBU. The maximum (a 3.5-fold) increase in biomass, noted at 1 μM m-CBU, occurred with an 83% decrease in the total Fl.

Thus, our study showed the effect of m-CBU on the growth of *S. orgaadayi* callus culture, which is probably due to the m-CBU effect on cell elongation and division. Dose-dependent changes in the accumulation of culture biomass (see Fig. 2) occurred together with a change in the rate of cells of various sizes and shapes (see Fig. 3, 4). The action of m-CBU at the lowest concentration (0.01 μM) reduced GI_{dry}, which could be due to a decrease in the volume of large cells by 23% with an equal control of the ratio of cell groups ranked by size. The highest

GI_{wet} and GI_{dry} observed under the action of 1 μM m-CBU were accompanied by an increased frequency of medium-sized and large cells and a decrease in the proportion of small cells (see Fig. 3, B). The concentration of 0.1 μM m-CBU had a lesser effect than 1 μM m-CBU, which manifested itself in an increase in the frequency of small cells and, accordingly, in a decrease in the proportion of medium-sized and large cells.

The highest stimulation of callus growth occurs under the influence of 0.1-10 μM m-CBU, leading to abundant small round meristematic cells. Growth acceleration results in a change of secondary metabolism and 80-95% lower total Fl ($p < 0.05$) compared to the control (see Fig. 5). At a high concentration (100 μM), m-CBU significantly stimulates culture growth without a noticeable change in the Fl level. Dose-dependent patterns of callus growth and Fl content under the influence of m-CBU may probably indicate a change in the functioning of enzymes involved. Goncharuk et al. [21] who studied the effect of exogenous cadmium and glyphosate on the phenolic compounds also indicate modification of FLs in cell culture: the sum of phenolic compounds increased when phenylpropanoids and flavonoids decreased.

FLs which could affect callus growth represent a highly diverse class of polyphenolic secondary metabolites possessing various properties. Along with catechins and quercetin known for their antioxidant activity, some FLs at high doses exert pro-oxidant effect and can damage cells [8].

FLs are involved in cell cycle regulation [22]. Possible mechanisms include the direct interaction of Fls (for example, quercetin) with Raf and MEK protein kinases which determine the transmission of mitotic signals, or their binding to the AhR receptor and the formation of a complex with ARNT which stimulates the transcription of the cell cycle inhibitor CDKNB1 [22, 23].

In addition, an indirect effect of FLs on growth due to changes in the transport of auxins and their metabolism is also possible. It is known that the spatiotemporal distribution of auxins, determined by the mechanisms of polar transport, plays a decisive role in their physiological effects [24]. Using *tt4* mutants as an example, it was shown that Fl deficiency enhances the flow of IAA [25]. Flavonols are thought to directly modulate IAA transport, a process that is reduced in the F39H-defective *tt7* mutant that overproduces kaempferol [25]. Non-glycosylated kaempferol and quercetin compete with the auxin transport inhibitor 1-N-naphthylphthalamic acid for a high-affinity binding site for a complex containing proteins PGP1, PGP2, and MDR1/PGP19 which belong to ATP-binding cassette transporters. FLs also act as regulators of IAA oxidase involved in the IAA degradation. FLs with o-hydroxyls in the B ring (quercetin, myricetin, luteolin) inhibit the enzyme activity while FLs with p-hydroxyls (apigenin, naringenin, naringin) stimulate it [26]. As a result, the first group of Fls are stimulates plant growth while the second group inhibits it.

Fl oxidation by plant peroxidases or other enzymes is an integral stage of normal plant growth and development [27, 28]. Due to oxidation, FLs possess prooxidant properties and can damage biological structures, which reduces cell viability [28].

The m-CBU can regulate the activity of heme-containing enzymes of the cytochrome P-450 (CYP) family of monooxygenases in humans. Enzymes of this group are found in all kingdoms and catalyze various chemical reactions [13, 25]. Changes in the *S. orgaadayi* cell culture growth and the Fl content influenced by m-CBU can probably also be explained by the regulation of the functioning of CYPs involved in Fl metabolism [2]. It has been shown that *CYP82D* encodes flavone-6-hydroxylase and 7-demethylase and is responsible for the biosynthesis

of Fls in sweet basil, while *CYP75* regulates the expression of flavonoid-3′-hydroxylase and flavonoid-3′,5′-hydroxylase which are involved in the synthesis of most anthocyanins in red grapes [13]. CYP93G1, the closest homologue of CYP93G2 in rice, is flavone synthase II (FNSII) which catalyzes the direct conversion of flavanones to flavones [30].

Plant hormones could act as other endogenous *S. orgaadayi* cell culture growth regulating factors the metabolism of which is also regulated by CYP. For example, CYP79B2/B3 is responsible for the conversion of L-tryptophan (L-Trp) to indole-3-acetaldoxime in an alternative L-Trp-dependent auxin biosynthesis pathway [13], cytokinin hydroxylase CYP735A1 and CYP735A2 catalyze the biosynthesis of trans-zeatins, and CYP72C1 inactivates brassinosteroids in *Arabidopsis thaliana* [13, 25].

Membrane mechanisms of m-CBU action on plant cells are also possible, by analogy with human cells. Such mechanisms may involve the blockade of fast neuronal sodium channels, which limits the propagation of the electrical potential along biological membranes. As a result, m-CBU stabilizes the concentration gradient of ions by regulating the water-electrolyte balance and prevents changes in the membrane permeability and transmembrane potential of the cell. Also, m-CBU enhances microsomal oxidation, showing a detoxifying effect in cells.

In our experiment, high Fls in 30-day subculture caused the completion of *S. orgaadayi* callus active growth in the control, which is consistent with the data on an increase in Fl accumulation at the stationary stage of cell culture [11, 12] and in leaves of *Lactuca sativa* L. plants that have completed their growth [5]. It has also been shown [12, 29] that in long-term suspension culture, the oxidative status (ROS accumulation) increases, and activation of antioxidant enzymes and secondary metabolites occurs. It should be expected that m-SBU increases the biomass of the cell culture, probably due to a lower level of F1 and, consequently, a longer time of active cell growth.

Our findings and other data [2, 12, 25, 28-30] allow us to assume the influence of m-SBU on the activity of enzymes that regulate the content of Fls and plant hormones, as well as enzymatic antioxidants.

Thus, the slow-growing *Saussurea orgaadayi* callus culture derived from the cotyledon explant produces flavonoids. Meta-chlorobenzhydryl urea (m-CBU) added to the growth medium at a concentration of 0.01 to 10 μM decreases the content of secondary metabolites in the callus. A lower level of flavonoids activates the growth in plant cell culture. Callus biomass increases with maximum growth index at 1 μM m-CBU. A further increase in the m-CBU concentration slows down the biomass growth. In the calli, the rate of cells of different types and sizes changes depending on the m-CBU concentration. Under the influence of 0.1 μm m-CBU, the number of small meristematic cells increased, and starting from 1 μM m-CBU, the round and oval cells of medium and large size are abundant while a proportion of small cells decreases. At 10 μM m-CBU, the cells were the largest in size, and at 100 μM m-CBU, the maximum number of large cells appeared. These data indicate acceleration of cell division at low m-CBU concentrations and cell elongation at medium and high m-CBU concentrations. Our findings show a dose-dependent effect of m-CBU on cell growth through a change in the content of flavonoids.

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