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The use of wheatgrass (*Thinopyrum intermedium*) in breeding

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Abstract. Wheatgrass (*Th. intermedium*) has been traditionally used in wheat breeding for obtaining wheat-wheatgrass hybrids and varieties with introgressions of new genes for economically valuable traits. However, in the 1980s in the United States wheatgrass was selected from among perennial plant species as having promise for domestication and the development of dual-purpose varieties for grain (as an alternative to perennial wheat) and hay. The result of this work was the creation of the wheatgrass varieties Kernza (The Land Institute, Kansas) and MN-Clearwater (University of Minnesota, Minnesota). In Omsk State Agrarian University, the variety Sova was developed by mass selection of the most winter-hardy biotypes with their subsequent combination from the population of wheatgrass obtained from The Land Institute. The average grain yield of the variety Sova is 9.2 dt/ha, green mass is 210.0 dt/ha, and hay is 71.0 dt/ha. Wheatgrass is a crop with a large production potential, beneficial environmental properties, and valuable grain for functional food. Many publications show the advantages of growing the Kernza variety compared to annual crops in reducing groundwater nitrate contamination, increasing soil carbon sequestration, and reducing energy and economic costs. However, breeding programs for domestication of perennial crops are very limited in Russia. This paper presents an overview of main tasks faced by breeders, aimed at enhancing the yield and cultivating wheatgrass efficiency as a perennial grain and fodder crop. To address them, both traditional and modern biotechnological and molecular cytogenetic approaches are used. The most important task is to transfer target genes of *Th. intermedium* to modern wheat varieties and decrease the level of chromatin carrying undesirable genes of the wild relative. The first consensus map of wheatgrass containing 10,029 markers was obtained, which is important for searching for genes and their introgressions to the wheat genome. The results of research on the nutritional and technological properties of wheatgrass grain for the development of food products as well as the differences in the quality of wheatgrass grain and wheat grain are presented.
Key words: perennial crop; wheat; domestication; selection; genes; ecology.

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Использование пырея среднего (*Thinopyrum intermedium*) в селекции

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Аннотация. Пырей средний (*Th. intermedium*) традиционно применялся в селекции пшеницы для получения пшенично-пырейных гибридов и сортов с интрогрессиями новых генов хозяйственно ценных признаков. Однако в 1980-х гг. в США из множества многолетних видов растений пырей был выбран для доместикации с целью создания сортов двойного направления использования – на зерно (альтернатива многолетней пшенице) и сено. В результате были выведены сорта пырея Kernza (The Land Institute, Канзас) и MN-Clearwater (Миннесотский университет, Миннесота). В Омском ГАУ из популяции *Th. intermedium*, полученной из The Land Institute, массовым отбором наиболее зимостойких биотипов с последующим их объединением создан сорт Сова. Средняя урожайность зерна сорта Сова составляет 9.2 ц/га, зеленой массы – 210.0 ц/га, сена – 71.0 ц/га. Пырей средний – культура с большим производственным потенциалом, полезными экологическими свойствами и ценным зерном для функционального питания. Во многих публикациях показаны преимущества возделывания сорта Kernza по сравнению с однолетними культурами: сокращение стока нитратов в грунтовые воды, увеличение секвестрации почвенного углерода, снижение энергетических и экономических затрат. Однако в России селекционные программы, направленные на доместикацию многолетних культур, весьма ограниченны. В настоящем обзоре рассматриваются основные задачи, стоящие перед селекцией и направ-

ленные на повышение урожайности зерна и эффективности возделывания пырея среднего в качестве многолетней зерновой и кормовой культуры. Для их решения используются как традиционные, так и современные биотехнологические и молекулярно-цитогенетические подходы. Важнейшей задачей считается передача целевых генов *Th. intermedium* в современные сорта пшеницы и сокращение дозы хроматина, несущего гены нежелательных признаков дикорастущего сородича. Получена первая консенсусная генетическая карта пырея среднего, содержащая 10 029 маркеров и представляющая интерес для поиска ценных генов и их интродукции в геном пшеницы. Представлены результаты исследований по оценке питательных и технологических свойств зерна пырея и полученных из него продуктов питания в сравнении с пшеницей.
Ключевые слова: многолетняя культура; пшеница; доместикация; отбор; гены; экология.

Introduction

Climate change is an urgent problem affecting food security, since in the arid agricultural landscapes of Africa, Asia, and the South America cereals yield is sharply decreasing, in particular that of maize, wheat, and sugar beetroot (IPCC…, 2019).

The traditional agricultural system based on the cultivation of annual crops implies the usage of pesticides and moldboard plow tillage, which significantly reduces its fertility, leads to erosion of arable land, leaching of nutrients, and carbon emissions (Stavridou et al., 2016; Vico, Brunsell, 2018). About 70 % of total greenhouse gas emissions (CO_2, CH_4, N_2O, etc.) account for the application and production of nitrogen fertilizers, 10–15 % – for agrotechnical methods of tillage, the rest – for the usage of pesticides and growth regulators (Berry et al., 2010).

Annual crops occupy more than three quarters of the crops area in the world according to the latest data, so an important element of regenerative agriculture is creating a rational structure of cultivated areas and increasing the biodiversity of cultivated crops (de Oliveira et al., 2019). In the coming decades, the expansion of cultivated areas under perennial crops, in addition to annual crops, will create opportunities for transitioning agriculture to a more sustainable development trajectory, reduce production costs, and improve the agrocenoses state (Amaducci et al., 2016).

Perennial crops have a longer growing period, due to which the soil is covered with vegetation longer, provide carbon accumulation in the soil, and reduce greenhouse gas emissions (Chimento, Amaducci, 2015; Schipanski et al., 2016). They have increased resistance to many negative biotic and abiotic environmental factors, form a powerful root system that improves plant water consumption and reduce nutrient losses in the soil (Zeri et al., 2013; Abraha et al., 2016). The wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey and oilseed culture *Silphium integrifolium* Michx. are examples of successful domestication of perennial crops. Wide hybridization of annual crops with perennial wild relatives is carried out in many scientific institutions and universities around the world to create such perennial crops as wheat, sorghum, rice, barley (Crews et al., 2018).

Biological and genetic properties of Intermediate wheatgrass (IWG)

Allohexaploid species *Th. intermedium* ($2n = 6x = 42$) (= syn. *Agropyron glaucum* (Desf. ex DC.) Roem. & Schult. = *Elytrigia intermedia* (Host) Nevski) is a perennial wild species characterized by a wide variety of morphological properties and high adaptability to biotic and abiotic stresses (Razmakhnin, 2008). This species is included in the tertiary gene pool, differs from all other species of the genus *Thinopyrum* A. Löve by high crossbreeding with bread wheat (average grain percentage of hybrids is 24) (Gill et al., 2006; Cui et al., 2018). However, the transfer of valuable genes from IWG to bread wheat is difficult, which is explained by limited recombination between chromosomes of these species in distant hybrids. Four main methods are used for targeted introgressions from the homeologous chromosomes of wild wheat relatives to the wheat genome: spontaneous translocations, radiation exposure, tissue culture, and induced homeologous recombination. The last method is used provided that the target gene is removed from the near centromeric regions where recombination is absent or difficult (Zhang P. et al., 2017).

The genomic composition of *Th. intermedium* (JJ^SSt) has been studied for decades. The results of genomic *in situ* hybridization (GISH) with usage of labeled DNA of different diploid species as probes showed that the J-genome is related to the genome of diploid species *Th. bessarabicum* and *Th. elongatum*, and the J^S-genome – modified form of the genome *Th. elongatum/Th. bessarabicum*. The St-genome is the main genome of perennial grasses; it shows great similarity with the genome of genus *Pseudoroegneria*, which is the most probable maternal parent of *Th. intermedium* (Chen et al., 1998; Chen, 2005; Mahelka et al., 2011; Kroupin et al., 2019).

In the 1930s, scientists had great expectations for wide hybridization, when N.V. Tsitsin in the Soviet Union, as well as other scientists in the USA and Canada, began to develop perennial wheat forms by crossing bread wheat with IWG (Suneson et al., 1963; Tsitsin, 1978). In the Main Botanical Garden named after N.V. Tsitsin of the RAS (Moscow), under the leadership of academician N.V. Tsitsin was formed a unique collection, which included the octoploid forms of wheat-wheatgrass hybrids (WWGHs) obtained using different species of wheatgrass, as well as varieties Istra 1, Zernokormovaya 169, Ostankino, Otrastayutshaya 38 (Upelniek et al., 2012). For the first time, the winter bread wheat varieties characterized by medium level of winter hardiness were created on the basis of wheat-wheatgrass hybrids WWGH 599 and WWGH 186. In the 1970s, the variety Zarya was developed in the Federal Research Center "Nemchinovka", which was cultivated on an area larger than 500 thousand hectares (Sandukhadze et al., 2021). Modern varieties and lines Multi 6R,

Lebedushka, Belyanka of Samara ARI have a substituted chromosome 6D(6Ag^{i}); varieties Tulaykovskaya 5, 10, 100 of Saratov ARI have a substituted chromosome 6D(6Ag^{i}2) with highly effective resistance genes to brown, stem, yellow rust, and powdery mildew belonging to *Th. intermedium* (Sibikeev et al., 2005; Salina et al., 2015). In Western Siberia, some perspective WWGHs based on *Th. intermedium* and *Ag. elongatum* were developed. They are recommended for inclusion in hybridization with varieties of winter and spring bread wheat in order to increase winter hardiness, resistance to rust diseases, and grain quality (Plotnikova et al., 2011; Razmakhnin et al., 2012). In China, since the early 1950s, systematic work has been carried out to increase wheat resistance to different abiotic and biotic environmental factors using *Th. intermedium*. The WWGHs with characteristics such as high winter hardiness, disease resistance, improved feed properties, and rapid post-harvest regrowth were involved in the breeding of perennial fodder wheat (Cui et al., 2018).

Biotechnological and molecular cytogenetic approaches to transfer the target gene to modern wheat varieties and reduce the unwanted alien chromatin of wild wheat relative are used (Kroupin et al., 2019). The genes of resistance to leaf, stem, yellow rust, powdery mildew (*Lr38*, *Sr44*, *Yr50*, *Pm40*, and *Pm43*), barley yellow dwarf virus (*Bdv2*, *Bdv3*), and wheat striped mosaic (*Wsm1*) were transferred to the wheat genome from IWG (Martynov et al., 2016; Ryan et al., 2018; Sibikeev et al., 2018).

Molecular markers for the analysis of the *Th. intermedium* genome, which makes it possible to purposefully transfer wheatgrass genes into the wheat genome, were developed (Kroupin et al., 2011; Li et al., 2016; Sibikeev et al., 2017). In particular, molecular markers have been developed to identify wheatgrass genes in the wheat genome: CAPS-marker for the *Vp-1* gene is used in breeding to increase resistance to pre-harvest sprouting (Divashuk et al., 2011; Kocheshkova et al., 2017); CAPS-marker P22F/PRa/PvuII for the *DREB1* gene, for the wheat drought tolerance breeding (Pochtovyi et al., 2013); molecular and cytogenetic markers specific to wheatgrass chromosome 1St#2, for breeding to increase the protein and gluten content in wheat grain (Li et al., 2013, 2016); WXTH-marker for the *Wx* gene, for changing starch composition and technological properties (Klimushina et al., 2020); PLUG, SCAR and *Thi*-GBS-markers, for identifying the chromosomes of the J-, J^{S}-, and St-genomes of wheatgrass (Hu et al., 2012; Tang et al., 2020; Qiao et al., 2021).

Along with molecular markers, cytogenetic markers are effectively used to identify chromosomes and their segments belonging to *Th. intermedium*, which are associated with agronomic traits (Yu et al., 2019; Nikitina et al., 2020). The oligosondes (*GAA*)10, *pSt122*, *pSc119.2-1*, *Oligo-B11*, *Oligo-pThp3.93*, *pAs1-1*, *pAs1-3*, *AFA-4* of the fluorescent (FISH) and genomic (GISH) hybridization are used to visualize *Th. intermedium* chromosomes in WWGHs and introgressive lines (Li et al., 2016; Xi et al., 2019; Wang et al., 2021). Three cytogenetically markers of tandem repeats, which were specific to *Th. intermedium* chromatin on different chromosomes of introgressive lines tolerant to phosphorus deficiency were developed (Zhang X. et al., 2021). The presence of reliable markers for wheatgrass chromosomes expands experimental possibilities for using this cereal in wheat breeding.

In 2016, the first consensus genetic map of IWG was obtained. It consists of 10,029 markers, each of 21 linkage groups contains between 237 and 683 markers with an average distance of 0.5 cM between each pair of markers (Kantarski et al., 2017). This map is of interest for identification of genes that control economically important agronomic traits and their introduction into the wheat genome. A total of 111 QTLs were detected for 17 variable traits in the M26 × M35 family including several large-effect QTLs responsible for seed retention, plant height, seed weight, seed threshing, and other economically important agronomic traits. By the method of association-mapping, 33 QTLs that control the grain size and weight were detected. When performing the selection of forms for seed weight, it was observed that the frequency of favorable QTL alleles in the IWG population was increased to >46 % (Larson et al., 2019).

Breeding programs for the wheatgrass domestication

Domestication of a new species is a risky and unpredictable process, because during selection for target traits, one cannot be sure how other traits, desirable or undesirable for breeding, will change. In the 1980s, at the Rodale Research Center (Kutztown, USA), IWG was selected for domestication and seed production from over 100 perennial species. Among perennial crops, this cereal has relatively large seeds, moderate spike fragility, and good threshability, along with greater biomass and excellent quality of fodder (Wagoner, 1990; Becker et al., 1992). Two selection cycles according to agronomic characteristics and seed size were carried out. The perspective genets (clones) of wheatgrass were identified and transferred for further study to the Land Institute (Salina, Kansas, USA) (DeHaan et al., 2005; Cox et al., 2010).

At the Land Institute, the selection cycles began with the development of indices based on the characteristics: seed weight per plant, seed weight per spike, percent of the bare seed, thousand kernel weight, and disease damage. A population for over-pollination was formed corresponding to the indices in each selection cycle from 50–70 genets with the most favorable combination of traits. After two selection cycles, the grain yield per unit area increased by 77 %, and the seed weight, by 23 % (DeHaan et al., 2018). At the Land Institute and the University of Minnesota (Minnesota, USA), the results of genome sequencing (Thinopyrummedium v2.1 DOE-JGI, https://phytozome-next.jgi.doe.gov/info/Tintermedium_v2_1) were actively used for the domestication of *Th. intermedium* in order to replace time-consuming selection by phenotype by GWAS and bioinformatics methods (Bajgain et al., 2019; Crain et al., 2020, 2021).

As a result of many years of work at the Land Institute, the wheatgrass variety Kernza was developed (named after the residents of Kansas), used both for seed production, green mass, and hay (haylage). During the second year of the cultivating of the variety, there was an 86 % nitrate re-

duction in groundwater, and a 13 % increase in soil carbon sequestration compared to annual crops (Glover et al., 2010; Culman et al., 2013; DeHaan, Van Tassel, 2014; Pugliese et al., 2019). Kernza is practically not affected by diseases and pests, the crop requires fewer agrotechnical operations, such as nitrogen fertilizers, tillage, pre-sowing seed treatment, and fungicide protection, thereby reducing energy and economic costs (DeHaan et al., 2005; Pugliese et al., 2019).

During the cultivation period of Kernza in Kansas in 2012–2016, the nitrogen fertilization has changed over time, beginning with ~110 kg per ha in 2012 and gradually decreasing to ~80 kg per ha in 2016. For this period, carbon emissions were reduced from 513 to 121 g $C \cdot m^{-2}$. Over the whole study period, the total carbon fixed was ~50 % higher than the carbon lost via respiration. Based on the cumulative net ecosystem exchange data (NEE), it was found that the perennial wheatgrass represented a substantial carbon sink 590.4 g $C \cdot m^{-2}$ per year (de Oliveira et al., 2018).

A five-year cultivation of the wheatgrass variety Kernza had positive effect on the soil structure and yield of the following crops in the crop rotation: it increased the microbiological activity and soil microbiota diversity compared to the soil microbiota under maize harvested for silage (Jungers et al., 2019). In comparison with annual crops such as maize and wheat, the variety Kernza also had a higher ability of maintaining the water-use efficiency (WUE) and evapotranspiration (ET) – about 97 % throughout the whole growing season. This was achieved thanks to a strong root system and water uptake from deeper soil layers, which is an important mechanism of adaptation to water deficit conditions (Suyker, Verma, 2009; Abraha et al., 2015; Sutherlin et al., 2019).

In 2011, a joint breeding program for improvement of Kernza was launched between the Land Institute and the University of Minnesota, which contributed to the commercial interest emergence for this perennial cereal. It was developed as a synthetic population at the University of Minnesota, prioritising grain-type direction, MN-Clearwater (experimental designation MN 1504), which can be cultivated for biomass and forage. Among 2,560 IWG genets received from the Land Institute, seven parents were selected according to the following set of characteristics: days to heading, plant height, spike weight, percentage free grain threshing, seed weight, and biomass weight to create a synthetic population of MN-Clearwater. In variety trials across Minnesota, MN-Clearwater produced 696 $kg \cdot ha^{-1}$, the thousand kernel weight was 6 g. This is a short-stemmed variety (113 cm), which had a good threshability (63 %), and low stem fragility with minimal lodging during research years (Bajgain et al., 2020). Programs for domestication and improvement of such IWG traits as seed size, threshability, reduction of spike fragility, and plant height for increasing resistance to lodging and diseases are also implemented at the University of Manitoba (Canada), at the University of Utah (USA), and at the University of Agricultural Sciences (Uppsala, Sweden) (Cattani, Asselin, 2016).

The introduction of optimal doses of fertilizers and appropriate agricultural technology increase the wheatgrass yield. Thus, in the autumn sowing of IWG population of grain-type (TLI-C2), grain yield was highest during the first year in response to nitrogen fertilization – 961 $kg \cdot ha^{-1}$ and gradually decreased in subsequent production years (Jungers et al., 2017). The experience of American farmers shows that IWG can be cultivated without replanting for 4–6 years, making a net profit by reducing production costs. The area occupied by Kernza in the USA in 2014 was approximately 87 hectares and doubled to 170 hectares in 2016. For further growth of the areas occupied under this crop, information on optimal establishment practices, assessment of forage nutritive value, ways to maintain grain yields over years, and weed management is needed (Lanker et al., 2020).

The usage of IWG grain for increasing the nutritional and biological value of bread and baked goods

An important aspect of the popularization of IWG in America and Europe was the use of Kernza grain for food production (Zhang X. et al., 2017). Bakery products, crackers, cereals, snacks produced on the basis of wheatgrass grain have a sweet nutty taste. The companies General Mills and Patagonia Provisions produce the wheatgrass grain goods under the trademark Kernza®, which belongs to the Land Institute. Currently, these companies are expanding the markets for these products. A chain of Birch Wood cafes has been opened in Minneapolis, serving tortillas and pancakes baked from flour of wheatgrass Kernza (Springmann et al., 2018).

Studies have been conducted for evaluation of technological characteristics of wheatgrass grain. The results have been used for the development of food products. The IWG grain quality is not inferior to wheat grain, but at the same time, there are significant differences (Becker et al., 1991).

IWG is characterized by a high protein and fiber content in whole grain flour – 20 and 16.4 %, while in whole grain wheat flour their content is 13 and 11 %, respectively (Rahardjo et al., 2018). Protein has more essential amino acids compared to wheat, in particular, 1.4 times more cysteine and methionine (Becker et al., 1991). The results of a 3-year research on the IWG variety Sova (*Th. intermediate*) under conditions of the southern forest-steppe of Western Siberia showed that the protein content in grain varied from 18.5 to 20.5 %. For the third year of the variety's production, the protein content increased by 2 %. This is probably related to an increase in the total number of important agronomic groups of microorganisms in the rhizosphere under the variety Sova, development of more powerful root system, and weather conditions (Shamanin et al., 2021).

Wheatgrass glutenin proteins contain fewer high-molecular-weight glutenin subunits (HMW-GS), which are similar in structure to wheat HMW-GS (67–120 kDa), but have a lower weight – 45–90 kDa (Zhang X. et al., 2014). The deficiency in HMW-GS with a molecular weight of >60 kDa in wheatgrass grain causes a weak gas-holding capacity and dough elasticity, which, in turn, leads to low bread making quality (Marti et al., 2016).

Due to the small size of wheatgrass seeds, they contained significantly less starch (46.7 %) compared to wheat (72 %), as well as more albumin and globulin proteins in the aleu-

rone layer. However, during domestication, the seed weight was increased by 23 % (DeHaan et al., 2018), which led to an increase in the endosperm proportion in the seed and, accordingly, starch. The technological and digestive properties of starch depend on its content. The management of its components, amylose and amylopectin can be regulated using combinations of alleles of *Wx* genes in *Th. intermedium* (Klimushina et al., 2020). In contrary to wheat starch, wheatgrass starch has a higher proportion of long amylose chains, a lower gelatinization temperature, which reduces the starch viscosity and retrograde and makes it suitable for the production of baked goods with a lower glycemic index (Zhong et al., 2019). *Th. intermedium* grain can also be used in a mixture with hard red wheat grain for the production of baked goods with a low glutenin content (Marti et al., 2015; Rahardjo et al., 2018).

Mixing wheatgrass grain flour and durum wheat grain flour in a ratio of 50:50 contributes to a good balance between the functional characteristics and digestive properties of baked goods. Particularly, cookies made of wheatgrass grain flour had the same quality as cookies made of ordinary wheat flour. In addition, the increased content of dietary fibers and antioxidants in wheatgrass flour baked goods makes them especially useful for human health (Marti et al., 2016).

IWG variety Sova as alternative to perennial wheat

Omsk State Agrarian University initiated a study on the cultivation of perennial wheat samples obtained from the international CIMMYT collection and wheatgrass populations developed at the Land Institute. The city of Omsk became one of the sites among multilocation experiments of perennial crops germplasm, the results of which are presented in the article of R.C. Hayes et al. (2018). The variety Sova was developed by mass selection of overwintered biotypes from the *Th. intermedium* population received from the Land Institute. Several selection rounds were carried out on the basis of traits of winter hardiness and spike productivity. The productivity components of 100 spikes were evaluated according to the following characteristics: spike weight and length, the number of spikelets and grains per spike, the number of grains per spikelet, grain weight per spike. A synthetic population adapted to the conditions of the southern forest-steppe of Western Siberia was formed by directed pollination of the selected biotypes. In 2020, the large-grain wheatgrass variety Sova was included in the State Register of Breeding Achievements Allowed for Use and recommended for cultivation in all regions of Russia (Fig. 1, 2).

The variety Sova can be cultivated as a dual-use crop – for grain and forage. The average grain yield was 0.92 t/ha, green mass – 21.0 t/ha, and hay – 7.1 t/ha (Shamanin et al., 2021). Omsk State Agrarian University produces original seeds of the variety Sova with subsequent reproduction of the elite category seeds in three basic farms of Omsk State Agrarian University: "Triticum", "Niva", and "Govin". In 2020, about 5 seed tons of the variety Sova were produced for farmers in the Omsk region. The average grain yield in the southern forest-steppe and steppe zones of the Omsk region was 0.4–0.6 t/ha.

Fig. 1. Variety Sova of the 2nd year reproduction in JSC "Niva" of Pavlograd region, Omsk oblast, 2020.

Fig. 2. Grain of spring bread wheat *Triticum aestivum* L. variety Pamyati Azieva (*a*) and grain of wheatgrass *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey variety Sova (*b*), experimental field of Omsk SAU.

Despite some progress made in the implementation of individual breeding programs, there are many tasks that require further selection solutions to improve the efficiency of wheatgrass cultivation as a perennial grain crop. First of all, it is necessary to increase the yield of wheatgrass grain. The grain yield of wheatgrass is lower than that of spring wheat, because part of its energy is spent on the development of the root system and branching after overwintering. A further increase in the wheatgrass grain yield can be achieved by repeated selections of forms with a smaller plant length and a smaller number of grains per spike, which seems advisable to increase the thousand kernel weight (TKW). This is evidenced by the research results, in which a negative correlation between the TKW and the plant height ($r = –0.3$, $p = 0.05$), between the TKW and the number of grains per spike ($r = –0.5$, $p = 0.01$) was noticed (Shamanin et al., 2021). The usage of genomic technologies and molecular mapping for the selection of genotypes with valuable traits will greatly contribute to improving the efficiency of breeding for increasing the grain yield of this perennial crop.

Efficient seed production technologies and agrotechnical methods of wheatgrass cultivation in specific agro-climatic zones are also a reserve for increasing the yield of this crop. For producing bread and bakery goods made of wheatgrass grain with functional properties, it is necessary to develop technologies for the food industry and market the demand

for this product by the population, which will allow to form a stable demand for this crop on the market.

Conclusion

The above review of world research shows that IWG is a culture with great production potential, beneficial ecological properties and valuable grain for functional food. Cultivation of *Th. intermedium* and other perennial crops – sorghum, rice, barley, Silfium, meadow and pasture grasses in agriculture will provide not only ecological, but also social and economic benefits. This is also important due to challenges associated with the climate warming, the necessity to reduce the greenhouse effect, in agricultural production as well. The grain of IWG can be used for bakery and confectionery products with improved nutritional value, and the whole plant can be used for biomass, hay, and haylage. IWG has increased resistance to many negative biotic and abiotic environmental factors, forms a strong root system that improves plant water consumption, reduces nutrient losses in the soil and carbon emissions. The wheatgrass varieties Kernza and Sova developed at the Land Institute (USA) and at Omsk State Agrarian University (Russia) indicate good prospects for breeding improvement of this crop. Considering that the variety Sova is significantly inferior to cultivated annual cereals in grain yield, new breeding programs aimed to increase the thousand kernel weight and the manufacturability of cultivation in specific agro-climatic zones are needed. Active marketing and development of technologies for production of the wheatgrass grain for functional food production are necessary to popularize a new crop on the market.

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Improving the efficacy of potato clonal micropropagation by inoculation with the rhizosphere bacteria *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2

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Abstract. Sustainable development of agriculture depends on the provision of quality seeds to the market. Inoculation with plant-growth-promoting rhizobacteria in *in vitro* culture can be used to improve the growth efficacy and performance of microplants. We examined the effect of *in vitro* inoculation of microplants of the cultivars Nevsky and Kondor with the strains *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 separately and in combination. We examined the morphological variables of plant growth in *in vitro* culture and under *ex vitro* adaptation conditions; we also investigated the growth and performance of the plants in the greenhouse. The dependence of the inoculation efficacy on potato genotype, growth stage, and inoculum composition was ascertained throughout the experiment. *In vitro*, *A. baldaniorum* Sp245 alone and in combination with *O. cytisi* IPA7.2 promoted the formation of roots on the microplants of both cultivars and the growth of Nevsky shoots. During plant growth *ex vitro*, all growth variables of the Nevsky microplants were promoted by *O. cytisi* IPA7.2 alone and in combination with *A. baldaniorum* Sp245. In both cultivars grown in the greenhouse, shoot growth was promoted in most inoculation treatments. The survival ability of the Nevsky microplants in the greenhouse increased 1.7-fold under the effect of simultaneous inoculation. Inoculation of microplants with a combination of *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 increased the number of Nevsky minitubers 1.5-fold and the number of Kondor minitubers 3.5-fold. Inoculation with the tested strains can be used to promote the growth of microplants and increase the yield of minitubers in potato seed breeding for the production of healthy planting material.
Key words: *Solanum tuberosum* L.; *Azospirillum baldaniorum* Sp245; *Ochrobactrum cytisi* IPA7.2; plant-microbe associations, clonal micropropagation; plant growth efficacy; adaptability; *in vitro*; *ex vitro*.

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Повышение эффективности клонального микроразмножения картофеля при инокуляции ризосферными бактериями *Azospirillum baldaniorum* Sp245 и *Ochrobactrum cytisi* IPA7.2

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Аннотация. Устойчивое развитие сельского хозяйства зависит от обеспечения рынка качественными семенами. Инокуляция растений рост-стимулирующими ризобактериями в культуре *in vitro* может быть использована для повышения эффективности роста и продуктивности микрорастений при получении оздоровленного посадочного материала картофеля. Изучено влияние инокуляции *in vitro* штаммами *Azospirillum baldaniorum* Sp245 и *Ochrobactrum cytisi* IPA7.2 по отдельности и в консорциуме на микрорастения сортов Невский и Кондор. Оценены морфологические параметры роста растений в культуре *in vitro*, в условиях адаптации *ex vitro*, а также показатели роста и продуктивности растений в грунтовой теплице. На протяжении всего опыта была установлена зависимость эффективности бактеризации от генотипа картофеля, этапа культивирования и состава инокулята. Методом иммунофлуоресцентного анализа показано, что оба штамма бактерий успешно вступают во взаимодействие с клетками растений без антагонистического взаимного влияния. В культуре *in vitro A. baldaniorum* Sp245 и консорциум штаммов стимулировали образование корней на микрорастениях обоих сортов и

рост побегов сорта Невский. На этапе культивирования *ex vitro* на все ростовые показатели микрорастений сорта Невский положительно влияла инокуляция *O. cytisi* IPA7.2 и консорциум штаммов. При выращивании в теплице в большинстве вариантов инокуляции стимулировался рост побегов обоих сортов. Приживаемость растений сорта Невский в теплице повысилась под действием одновременной коинокуляции в 1.7 раза. Инокуляция микрорастений консорциумом штаммов *A. baldaniorum* Sp245 и *O. cytisi* IPA7.2 увеличивала количество мини-клубней у сорта Невский в 1.5 раза, а у сорта Кондор – в 3.5 раза. Инокуляция изученными штаммами может быть использована для стимулирования роста микрорастений и повышения урожайности мини-клубней в системе семеноводства картофеля при получении оздоровленного посадочного материала.
Ключевые слова: *Solanum tuberosum* L.; *Azospirillum baldaniorum* Sp245; *Ochrobactrum cytisi* IPA7.2; растительно-микробные ассоциации; клональное микроразмножение; эффективность роста растений; адаптационная способность; *in vitro*; *ex vitro*.

Introduction

In the production of seeds of many vegetatively propagated crops, *in vitro* clonal micropropagation methods have been widely used (Rajasekharan, Sahijram, 2015). In the clonal micropropagation of various plant species, rhizobacteria of different taxonomic groups can be used (Orlikowska et al., 2017; Soumare et al., 2021). Among herbaceous plants, orchid (Castillo-Pérez et al., 2021), sugarcane (Oliveira et al., 2002), and some other species (Dias et al., 2009) predominate as bacterization objects. Bacterial strains capable of promoting potato microclonal growth *in vitro*, adaptation to *ex vitro* conditions, and minituber productivity have been isolated (Oswald et al., 2010). Proper selection of bacterial associates is crucial (Wang et al., 2016). Our preliminary work has shown that pure cultures of the associative rhizobacteria *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 promote the growth of potato microplants *in vitro* and *ex vitro* (Tkachenko et al., 2015; Burygin et al., 2019; Kargapolova et al., 2020).

Some authors have pointed out that joint inoculation of plants with two or more strains of rhizospheric plant-growth-promoting bacteria (PGPR) can be more effective than inoculation with pure cultures (Thomas et al., 2010). When using consortia of strains for inoculation, one has to see that the component cultures are compatible (Yegorenkova et al., 2016). We have previously found that for strains with different characteristics, the inoculation of microplants during growth *in vitro* may be important (Burygin et al., 2018).

Here we examined the efficacy of inoculation of potato (*Solanum tuberosum* L. cvs. Nevsky and Kondor) microplants with pure cultures of *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 and with their mixture. The specific aim was to use clonal micropropagation to improve the production efficacy for seeds of healthy planting material.

Materials and methods

Growing of potato microplants *in vitro*. We used microplants of two middle early potato cultivars, Kondor and Nevsky. The cultivars had been obtained from the *in vitro* collection of the Department of Plant Breeding, Selection, and Genetics of the Faculty of Agronomy at Saratov State Vavilov Agrarian University (Saratov) and had been produced by isolation of apical meristems. The Nevsky and Kondor cultivars were used as material for study because the State Register for Selection Achievements Admitted for Usage (National List) (https://gossortrf.ru/gosreestr/) recommends them to be grown in the Lower Volga zone. Nevsky is a domestically bred cultivar (Russian Potato Research Center, Russia), whereas Kondor is a foreign-bred one (AGRICO U.A., Netherlands).

Microcuttings with one leaf and one bud were placed in a hormone-free liquid nutrient Murashige–Skoog medium (Murashige, Skoog, 1962). Plants were grown for 30 days at a temperature of 24 °C, a humidity of 60 %, a light intensity of 60 μM/($m^2 \cdot s$), and a day length of 16 h. The shoot and root morphometric variables examined were shoot length (mm), number of nodes per shoot, average root length (mm), and number of roots per shoot.

Inoculation of microcuttings. We used two rhizospheric bacterial strains – *A. baldaniorum* Sp245 (Baldani et al., 1983) and *O. cytisi* IPA7.2 (Burygin et al., 2017, 2019). Both strains were from the IBPPM RAS Collection of Rhizosphere Microorganisms (Saratov; http://collection.ibppm.ru/). Cultures were grown at 35 °C on a rotary shaker with a stirring intensity of 120 rpm until the end of the exponential growth phase (18 h) in a liquid malate medium composed as follows (g/l): Na malate, 5.0; KH_2PO_4, 0.4; NaCl, 0.1; $MgSO_4$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.002; NH_4Cl, 1.0, pH 6.8–7.0 (Döbereiner, Day, 1976). The cells were sedimented by centrifugation at 3000 g under sterile conditions and were resuspended in 0.12 M phosphate buffer (pH 7.2) containing (g/l): KH_2PO_4, 0.43; Na_2HPO_4, 1.68; NaCl, 7.2. Centrifugation was repeated twice in phosphate-buffered saline. For inoculation, 0.1 ml of cell suspension (10^8 cells/ml) was added to the tubes with plants, each tube containing 10 ml of the Murashige–Skoog medium. The final cell density in the medium was 10^6 cells/ml.

Bacteria were inoculated separately [*A. baldaniorum* Sp245 on day 0 of growth (microcuttings) and *O. cytisi* IPA7.2 on day 15 of growth] and in combination [simultaneously on day 15 of growth or successively (*A. baldaniorum* Sp245 on day 0 of growth and then *O. cytisi* IPA7.2 on day 15 of growth)]. The control was microplants grown without bacteria.

Growing of potato microplants *ex vitro*. Microplants were adapted to *ex vitro* conditions in a phytochamber in soil-filled vessels for 20 days (temperature, 24 °C; humidity, 60 %; light intensity, 60 μM/($m^2 \cdot s$); day length, 16 h). The morphometric variables analyzed were shoot length, leaf number, and leaf area.

Next, the plants were transferred to a soil-based greenhouse covered with agrotextile and were planted in a pattern of 0.4 × 0.4 m. The temperature and humidity in the greenhouse

were not regulated and depended on the weather; therefore, they were stressful for the plants (the daytime temperature could rise as high as 30 °C, and humidity could drop below 60 %). The plants were watered as needed (every 3–5 days on average). Three weeks after planting and at the beginning of the budding and flowering phase, we recorded the percentage of surviving plants, the height of the plants, the numbers of shoots and leaves, and the area of the leaves. Minitubers were dug out after the vines wilted. The number and weight of minitubers per plant and the weight and diameter of each tuber were recorded.

Immunofluorescence analysis. Bacteria on plant roots were identified by immunofluorescence analysis on day 30 after inoculation, by using strain-specific antibodies (Shelud'ko et al., 2010). The controls were uninoculated and inoculated roots treated with nonspecific antibodies. Nonspecific antibody sorption was blocked by 2-h incubation of root sections at room temperature in 0.05 % polyethylene glycol solution (MW 20000) in phosphate buffer. The primary antibodies were strain-specific rabbit antibodies to the LPS of *A. baldaniorum* Sp245 and to the LPS of *O. cytisi* IPA7.2 (concentration, 50 μg/ml); the secondary antibodies were tetramethylrhodamine isothiocyanate (TRITC)-labeled goat antirabbit antibodies (Abcam, USA; concentration, 1 μg/ml).

The inoculated roots of the microplants were observed with a TCS SP5 confocal microscope (Leica Microsystems, Germany) at the Simbioz Center for the Collective Use of Research Equipment in the Field of Physical-Chemical Biology and Nanobiotechnology (IBFRM RAS, Saratov).

Statistics. The experiment was repeated twice. In each experiment, three replicates of 10 plants each were used in each experimental treatment, with a total of 30 plants per treatment in each experiment. Data from all experiments were subjected to two-way analysis of variance (ANOVA) and were evaluated for a significance level p of 0.05. To test the null hypothesis, we calculated the F-test statistic (F_{fact}) and then determined the least significant difference ($LSD_{0.05}$) between experimental treatments. Means from each experimental treatment were subjected to multiple comparison by Duncan's test. The program package used was AGROS, a package for statistical and biometrical-genetic analysis in plant breeding and selection (version 2.09).

Results

Effect of bacteria on growth and development of potato microclones *in vitro*

For all variables examined *in vitro*, except for average root length, Kondor microplants lagged behind in growth, as compared with Nevsky microplants (Fig. 1). The shoot length of Nevsky microplants was promoted by all inoculation treatments (see Fig. 1, *a*). The microplants inoculated with *A. baldaniorum* Sp245 alone were 18.9 % taller than the control – the maximum height among the treatments examined. In Kondor, all inoculation treatments suppressed shoot length, except for the treatment with *A. baldaniorum* Sp245, in which the plants did not differ from the control.

In Nevsky, all inoculation treatments increased the number of nodes per shoot (see Fig. 1, *b*), except for inoculation with *O. cytisi* IPA7.2, when plant length did not differ from that in the control. The Nevsky microplants inoculated with *A. baldaniorum* Sp245 had 11.6 % more nodes than did the controls. The microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 5 % more nodes on their shoots than did the controls. The microplants inoculated simultaneously with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) had 10.5 % more nodes than did the controls. The values for the Kondor microplants inoculated with *A. baldaniorum* Sp245 alone and sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) were at the control level. The other inoculation treatments decreased the number of nodules on Kondor microplants.

In Nevsky, the average root length (see Fig. 1, *c*) increased by 4 % with *A. baldaniorum* Sp245 and by 3.7 % with *O. cytisi* IPA7.2, as compared with the control. However, sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) suppressed root length – it was 4.3 % lower than the control value. In Kondor, all inoculation treatments inhibited root length.

In both cultivars, the number of roots (see Fig. 1, *d*) increased after both inoculation with *A. baldaniorum* Sp245 alone and sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). With *A. baldaniorum* Sp245, both Nevsky and Kondor had 12.5 % more roots than did the control. The Nevsky microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 6.3 % more roots than did the control. The Kondor microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 26.7 % more roots than did the control.

Thus, inoculation with *A. baldaniorum* Sp245 alone and sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had positive effects on the Nevsky microplants. The shoot length, the number of nodes per shoot, and the number of roots increased, whereas the average root length decreased.

Identification of bacteria on roots of potato microplants *in vitro*

Immunofluorescence analysis of the Nevsky roots by using confocal microscopy showed that both strains interacted successfully with plant cells (Fig. 2).

Both strains were detected on roots after both inoculation with pure cultures and coinoculation. Both strains were present in coinoculation treatments, which indicates that there was no antagonism between them and that neither strain had any advantage over the other in interacting with root cells.

Effect of bacteria on adaptation of potato microclones *ex vitro*

The survival ability of the microplants formed *in vitro* in soil-filled vessels under phytochamber conditions (*ex vitro* stage) was high (more than 80 %) (Fig. 3, *a*). In Nevsky, the survival ability decreased by 6 %, as compared with the control, only after inoculation with *O. cytisi* IPA7.2 alone. In Kondor, the survival ability decreased by 11 % after coinoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) and by 14 % after inoculation with *O. cytisi* IPA7.2 alone.

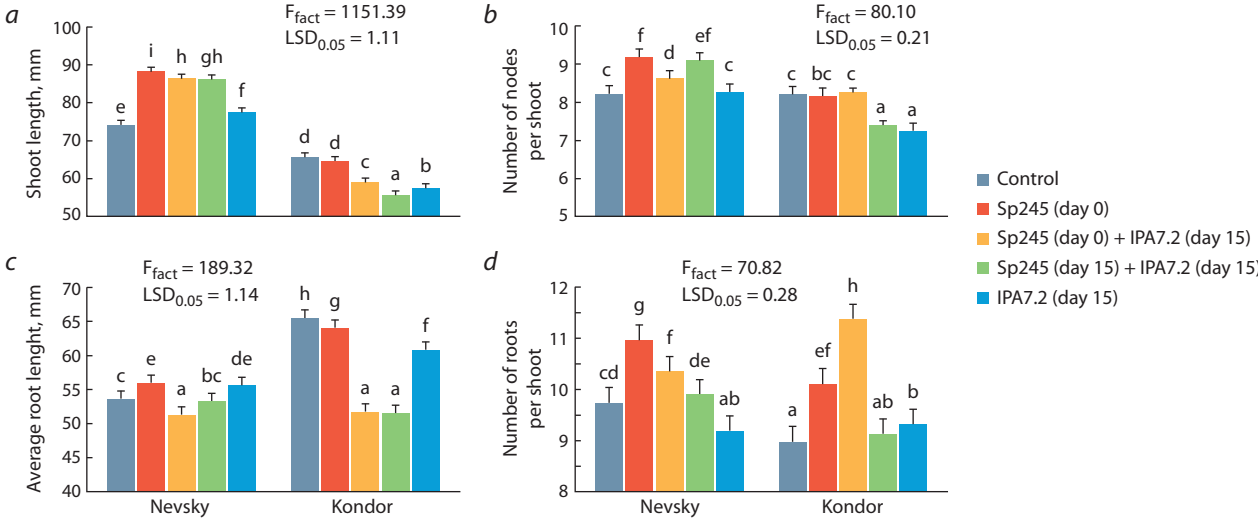

Fig. 1. Effect of *in vitro* inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on morphological variables of potato microplants: *a*, shoot length; *b*, number of nodes per shoot; *c*, average root length; *d*, number of roots per shoot.

Here and in the Figures 3–5: for all variables, a significance level *p* of 0.05 ($n = 30$) was used. Different Latin letters (a, b, c, etc.) indicate values from treatments that differ significantly according to multiple comparison by Duncan's test.

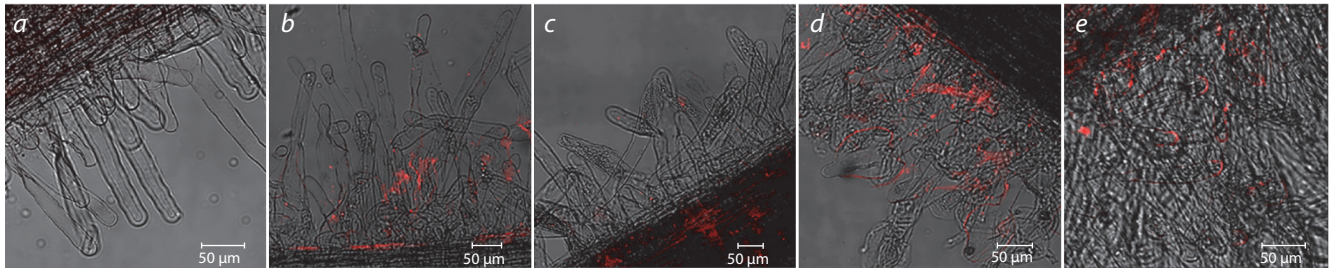

Fig. 2. Identification of bacteria on roots of potato microplants by immunofluorescence confocal microscopy: *a*, control (no inoculation), antibodies to *A. baldaniorum* Sp245 and antibodies to *O. cytisi* IPA7.2; *b*, inoculation with *A. baldaniorum* Sp245, antibodies to *A. baldaniorum* Sp245; *c*, coinoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, antibodies to *A. baldaniorum* Sp245; *d*, coinoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, antibodies to *O. cytisi* IPA7.2; *e*, inoculation with *O. cytisi* IPA7.2, antibodies to *O. cytisi* IPA7.2.

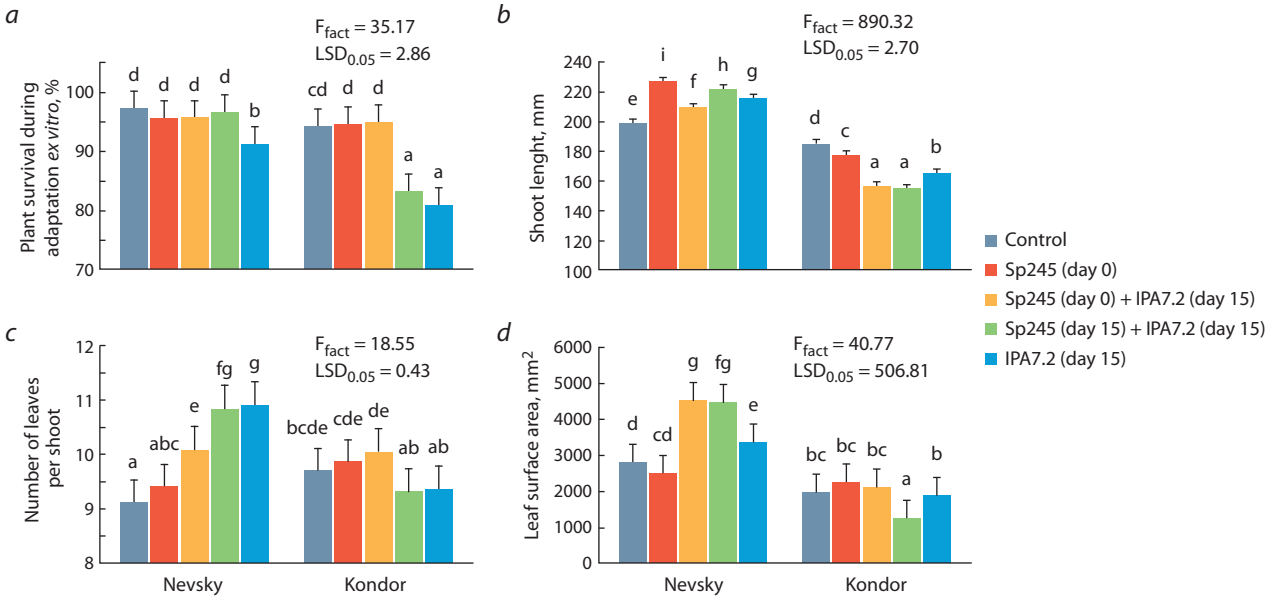

Fig. 3. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on morphological variables of potato microplants during plant adaptation *ex vitro*: *a*, plant survival during adaptation *ex vitro*; *b*, shoot length; *c*, number of leaves per shoot; *d*, leaf surface area.

Under *ex vitro* conditions, we found significant genotype effects on all variables examined. The Nevsky cultivar formed larger shoots with more large leaves than the Kondor cultivar (see Fig. 3).

In Nevsky, all inoculation treatments promoted shoot length (see Fig. 3, *b*). With *A. baldaniorum* Sp245 alone, shoot height increased by 14 %, as compared with the control; with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day15) inoculated sequentially, by 5 %; with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) inoculated simultaneously, by 11.5 %; with *O. cytisi* IPA7.2 alone, by 8 %. In Kondor, all inoculation treatments suppressed shoot length (by 4 to 16 %).

The number of leaves per shoot (see Fig. 3, *c*) did not differ from the control value in any of the experimental treatments in Kondor. In Nevsky, on the contrary, all inoculation treatments promoted this variable, except for the use of *A. baldaniorum* Sp245 alone, in which no significant differences from the control were found. The Nevsky plants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) formed 10.5 % more leaves than did the control plants. The Nevsky plants inoculated simultaneously with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) formed 18.7 % more leaves than did the control plants. The Nevsky plants inoculated with *O. cytisi* IPA7.2 alone had 19.4 % more leaves than did the control plants.

In Kondor, the leaf surface area (see Fig. 3, *d*) did not differ from the control value in any treatment except the simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15), in which a 36.6 % negative effect was recorded. In Nevsky, the leaf surface area was promoted with *O. cytisi* IPA7.2 alone and with *A. baldaniorum* Sp245 coinoculated with *O. cytisi* IPA7.2 (in both coinoculation treatments, the leaf surface area was 60 % larger than that in the control). The Nevsky plants inoculated with *O. cytisi* IPA7.2 alone had larger leaves (by 19 %) than did the control plants.

Thus, the effects of microplant inoculation under *in vitro* conditions and during *ex vitro* adaptation depended significantly on the plant genotype. In Nevsky, all variables were promoted with *O. cytisi* IPA7.2 alone and in combination with *A. baldaniorum* Sp245. In Kondor, the effect was negative, or the plants did not differ from the control ones.

Effect of bacteria on microplant growth in the soil-based greenhouse and on minituber yield

The survival ability of plants in the soil-based greenhouse was significantly lower than that in the vessels under controlled conditions (Fig. 4, *a*), because environmental factors were uncontrolled and depended on the surrounding milieu. In Nevsky, survival ranged from 30 to 64 %; in Kondor, it was even lower – 18.33 to 25 %. In Nevsky, plant survival in the soil-based greenhouse was promoted by inoculation with *O. cytisi* IPA7.2 alone (by 1.5 times) and with *O. cytisi* IPA7.2 combined with *A. baldaniorum* Sp245 (by 1.2 and 1.7 times). In Kondor, the inoculation results did not differ from the control values.

As in the previous stages, the Kondor cultivar had significantly less green matter than did the Nevsky cultivar. Under greenhouse conditions, the positive effect of inoculation was stronger than in the previous growing stages (see Fig. 4). In Kondor, shoot length was suppressed by 11 % only after inoculation with *O. cytisi* IPA7.2 alone. In Kondor, no significant effects were observed in two experimental treatments: *A. baldaniorum* Sp245 alone (shoot length) and *A. baldaniorum* Sp245 (day 0) combined with *O. cytisi* IPA7.2 (day 15) (leaf area). In the other treatments, inoculation led to positive effects.

In both cultivars, shoot length (see Fig. 4, *b*) was promoted by sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) (by 57.1 and 27.5 %, respectively) and by simultaneous inoculation (day 15) (by 60.6 and 13.8 %, respectively).

In both cultivars, the leaf number (see Fig. 4, *c*) was promoted the most after simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) (by 80.5 and 51.1 %, respectively).

In both cultivars, the leaf surface area (see Fig. 4, *d*) increased in most inoculation treatments, but the increase was greatest with *O. cytisi* IPA7.2 alone (by 71.0 % in Nevsky and by 41.0 % in Kondor).

Tuber size was promoted the most with *O. cytisi* IPA7.2 alone (in Kondor) and with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) inoculated sequentially (in both cultivars) (Fig. 5*a*, *b*). In Kondor, the larger minituber diameter was increased the most with *O. cytisi* IPA7.2 alone (by 41.9 %), and in Nevsky, it was increased the most with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) inoculated simultaneously (by 12.5 %).

In most experimental treatments, the weight of minitubers (see Fig. 5, *b*) did not differ from that in the control. In Nevsky, tuber weight was suppressed after inoculation with *A. baldaniorum* Sp245 alone (by 70.5 %) and after simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) (by 20.5 %). In Kondor, tuber weight increased by 48.7 % after inoculation with *O. cytisi* IPA7.2 alone (day 15). Thus, in Kondor, both size and weight of minitubers were promoted by *O. cytisi* IPA7.2.

In Kondor, the minituber yield was lower than that in Nevsky, in agreement with the morphometric variables in all previous stages (see Fig. 5, *c*). Yet, in the higher-yielding Nevsky cultivar inoculation increased the minituber yield to a lesser extent than in the lower-yielding Kondor cultivar. The positive effect of microplant inoculation *in vitro* was maximal after sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). In this inoculation treatment, the yield of minitubers per square meter increased by 11.1 % in Nevsky and 6.8-fold in Kondor.

In these experiments, between 2.67 and 9.33 tubers were obtained per plant (see Fig. 5, *d*). The number of tubers per plant did not differ significantly between cultivars. The sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) affected Nevsky and Kondor 3.5- and 1.5-fold, respectively. In Nevsky, the number of minitubers per plant was also increased with *A. baldaniorum* Sp245 alone. In Kondor, the number of minitubers per plant was also increased with *O. cytisi* IPA7.2 alone (1.9-fold).

Thus, sequential inoculation *in vitro* of microplants with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15)

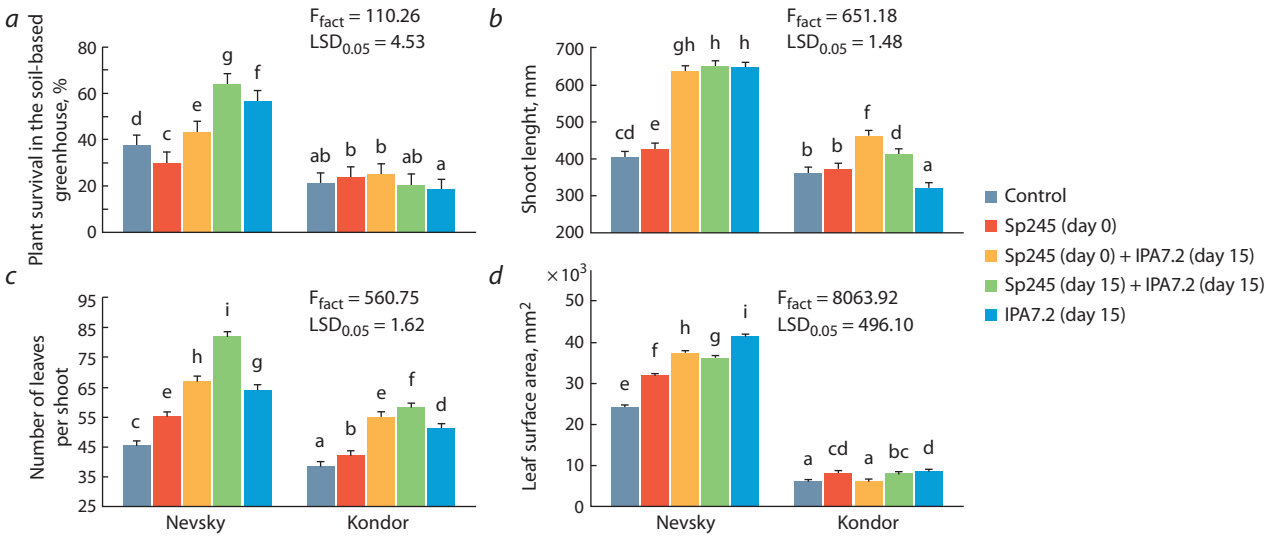

Fig. 4. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on variables of potato microplants grown in the soil-based greenhouse: *a*, plant survival in the soil-based greenhouse; *b*, shoot length; *c*, number of leaves per shoot; *d*, leaf surface area.

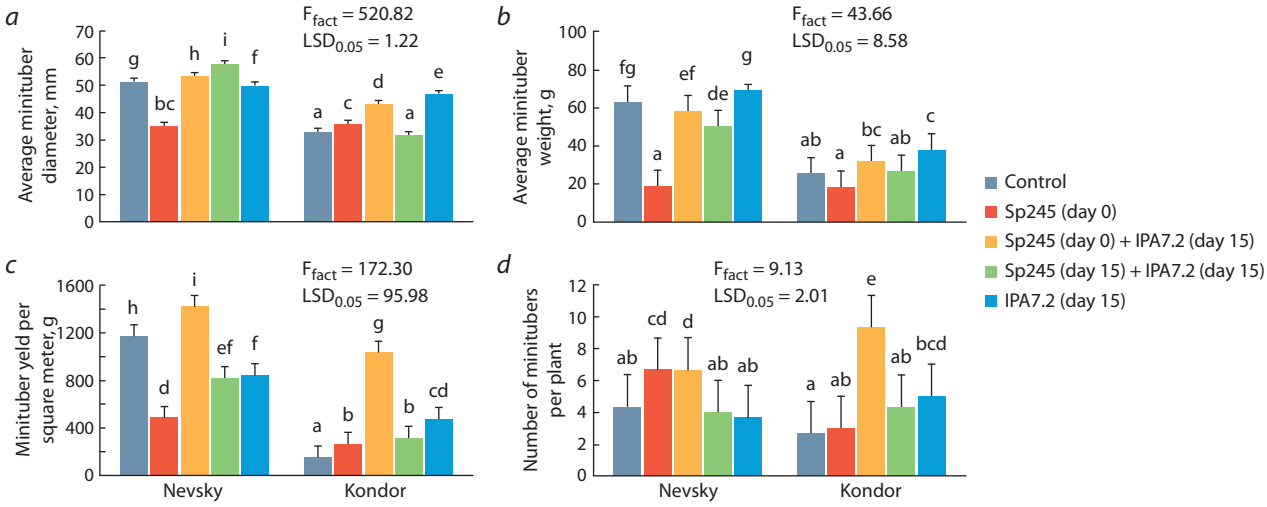

Fig. 5. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on minituber yield in potato plants grown in the soil-based greenhouse: *a*, average minituber diameter; *b*, average minituber weight; *c*, minituber yield per square meter; *d*, number of minitubers per plant.

significantly increased the weight and number of minitubers, which constitute healthy and unconventional planting material.

Discussion

Generation of healthy planting material is important in potato production technology. The clonal micropropagation of pathogen-free plants by growing apical meristems *in vitro* is obligatory in potato seed production. The effectiveness of this method can be increased by using rhizosphere bacteria. The data in the literature indicate that bacteria promote plant growth at all stages, including growth *in vitro* and *in vivo*, and they also promote the adaptive ability of microplants planted in nonsterile settings *ex vitro* (Oswald et al., 2010; Belimov et al., 2015; Santiago et al., 2017; Soumare et al., 2021).

Our previous studies have shown that the associative rhizospheric bacteria *A. baldaniorum* Sp245 (Tkachenko et al., 2015) and *O. cytisi* IPA7.2 (Burygin et al., 2019) can be used to promote the growth of potato microplants *in vitro* and *ex vitro*. The ability of *Azospirillum* bacteria to promote potato growth and productivity, including in the seed production system, is well known (Naqqash et al., 2016; Kargapolova et al., 2020; Tkachenko et al., 2021). The efficacy of use of these bacteria is higher *in vitro* (optimal conditions) but is lower when plants are grown in the field (Bacilio et al., 2017).

Our results also show that as compared to the other treatments, inoculation with *A. baldaniorum* Sp245 alone better stimulated the growth of Nevsky microplants *in vitro* (optimal conditions) than it did *ex vitro* or in soil under greenhouse conditions. *A. baldaniorum* Sp245 was isolated from wheat roots (Baldani et al., 1983; Ferreira et al., 2020) and is a model for many studies. Our data show that this strain has a high ability to produce the plant hormone indole-3-acetic

acid, which explains its promotion of the growth of microplant roots (Kargapolova et al., 2020).

O. cytisi IPA7.2, which we isolated directly from potato roots and which is native to the soils of Saratov Region, is more resistant to stress than *Azospirillum* (Burygin et al., 2017, 2019). This strain withstands large fluctuations in temperature and high salt and herbicide, which explains its ability to protect plants from stress, including osmotic stress (Evseeva et al., 2019).

The effect of bacterial inoculation on the formation and linear growth of plant organs (shoots and roots) depends on the hormonal balance existing in the plant at the moment. This balance is determined by genetic features, environmental factors, and the changes that specific strains cause in it (Arkhipova et al., 2020). Therefore, the promotion of shoot growth does not always coincide with that of root growth, and the effect of different strains may differ for different plant genotypes.

Combining different strains (e. g., azospirilla with other microsymbionts) for plant inoculation is considered promising owing to the possible synergistic effect and to the greater stability of the multicomponent system (Panahyan-e-Kivi et al., 2016; Trdan et al., 2019; Gavilanes et al., 2020). But in coinoculation, the compatibility of different strains and their ability to coinhabit plants without causing antagonism is important (O'Brien, Harrison, 2021). The efficacy of inoculation depends on plant genotype, development stage, and external and internal conditions (Andreote et al., 2010).

Previous work by us has found that the inoculation stage depends on the characteristics of the strain used (Burygin et al., 2018). *A. baldaniorum* Sp245 cannot grow independently on a nutrient growth medium for microplants and therefore can be used for inoculation at any stage of microplant growth *in vitro*. *O. cytisi* IPA7.2 can grow intensely on a nutrient growth medium for microplants and therefore can be used for inoculation only in the second half of the culturing period. Therefore, we examined two options for coinoculating microplants with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2: simultaneous inoculation (day 15 of growth) and sequential inoculation (*A. baldaniorum* Sp245 on day 0 and *O. cytisi* IPA7.2 on day 15 of growth). Our results show that *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 can be simultaneously present on potato roots without being antagonistic to each other (see Fig. 2). Synergistic effect, however, was not observed in all treatments, and it depended on the growth stage and the potato cultivar. Under *in vitro* conditions (see Fig. 1), the effects of the coinoculation treatments on most variables were not greater than the effect of each strain used separately, including *A. baldaniorum* Sp245, which was a good promoter of the growth of the Nevsky microplants.

In coinoculation, the adaptation ability of the microplants under favorable laboratory conditions at the stage of planting *ex vitro* (see Fig. 3) remained at the control level or at the level of the effects produced by the strains separately. But under the stressful conditions of the soil-based greenhouse, including poorly controlled environmental factors, the protective effect of inoculation was more pronounced (see Fig. 4), at least in Nevsky. In particular, after simultaneous inoculation, the survival ability of the Nevsky plants increased the most (by 71 %) – an effect greater than the positive effect of inoculation with *O. cytisi* IPA7.2 alone by almost 20 %.

Positive effect of coinoculation on the number and area of leaves was observed for the Nevsky cultivar during adaptation *ex vitro* (see Fig. 3), and the effect on the leaf area was synergistic. The promoting effect of inoculation was maximal under unfavorable greenhouse conditions (see Fig. 4), in agreement with the data of Cesari et al. (2019), who reported increased plant tolerance to stress under the influence of inoculation, including coinoculation with a bacterial consortium containing azospirilla. Coinoculation promoted the growth variables of both cultivars at the same level or even greater than did inoculation with *O. cytisi* IPA7.2 alone.

The efficacy of the whole technology of production of healthy potato planting material ultimately depends on the yield of minitubers. In seed breeding, it is not so much the weight of minitubers that matters as it is their number on plants, because the number of minitubers per plant determines the coefficient and rate of seed multiplication. The effect of inoculation on minituber production was particularly strong and was evident in both cultivars (see Fig. 5). The average minituber size changed nonsignificantly, but the number of minitubers on the plants increased significantly after sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). In Nevsky, the yield of minitubers was increased 1.5-fold; in Kondor, 3.5-fold. In Kondor, the sequential inoculation had a synergistic effect, as compared with the effects of the strains used separately. Similar synergistic effect on the yield of minitubers per square meter was noted in both cultivars in the same inoculation treatment.

The effects of sequential inoculation with *A. baldaniorum* Sp245 (microcuttings, day 0) and *O. cytisi* IPA7.2 (day 15) differed from those of simultaneous inoculation (day 15) at different stages of microplant growth. However, considering the promoting effect of *A. baldaniorum* Sp245 *in vitro* and the final yield of minitubers, sequential inoculation can be regarded as preferable.

Conclusions

Analysis of the experimental data shows that the bacterial strains *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, both individually and in combination, had a positive effect on potato microplants. This effect manifested itself differently at different stages of plant growth. The maximal positive effect of inoculation *in vitro* was that on the number of adventitious roots; the number and area of leaves (during plant adaptation *ex vitro*); and the weight of minitubers and all variables for the vegetative portion of shoots (during plant growth in the soil-based greenhouse). The two strains were not antagonistic to each other. The growth-promoting effect of the bacteria depended significantly on the potato genotype. The positive effect of the interstrain interaction was maximal when plants were grown in the open ground. The strains *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, separately and in combination, can be recommended as inoculants for *in vitro*-grown potato microplants in potato clonal micropropagation to produce healthy planting material.

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Psycho-emotional stress, folliculogenesis, and reproductive technologies: clinical and experimental data

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Abstract. Modern life, especially in large cities, exposes people to a high level of noise, high density of population, disrupted sleeping, large amount of excessive and controversial information as well as to other negative factors; all this may cause chronic psycho-emotional stress. The latest publications often use the term "Syndrome of megalopolis", which means disruption of sleeping, high anxiety, and altered reproductive function. Medical treatment of infertility may also be considered as a stress factor, especially when infertility lasts for years and is aggravated with emotional frustration. Long-lasting distress may worsen health in general and suppress reproductive function, in particular. The review presents the data on the effects of maternal stress on folliculogenesis, especially when assisted reproductive technologies (ARTs) are used. Clinical data are presented alongside data from laboratory animal experiments. Different maternal stress models are taken into account in respect of their influence on oocyte maturation and embryo development. The interfering of psycho-emotional stress and reproductive function is the focus of the review. In these situations, exogenous hormones compensate for the stress-related disruption of the hypothalamic-pituitary-gonadal axis. When ARTs are implemented, stress-induced disruption of oogenesis is realized not via a decrease in hypothalamic and pituitary hormones, but by other ways, which involve paracrine mechanisms described in this review. Based on the literature analysis, one may conclude that stress negatively affects oocyte maturation in the ovary and suppresses subsequent embryo development. The role of some ovarian paracrine factors, such as BDNF, GDF-9, HB-EGF, TNF-α, and some others has been elucidated.
Key words: stress; long-term effects; folliculogenesis; assisted reproductive technologies; preimplantation embryo.

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Психоэмоциональный стресс, фолликулогенез и репродуктивные технологии: клинические и экспериментальные данные

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Аннотация. Темп современной жизни, уровень шума, плотность населения, особенно в больших городах, нарушение ритма сна и отдыха, количество получаемой и обрабатываемой разнонаправленной информации и другие факторы современной жизни в мегаполисах могут приводить к хроническому психоэмоциональному стрессу. Все чаще в публикациях последних лет появляется понятие «синдром мегаполиса», объединяющее нарушения сна, проявления тревожности и изменение репродуктивной функции. Медицинские процедуры также считаются стрессовыми факторами; более того, само состояние бесплодия, как и его лечение, нередко является затяжным, длится годами, что в свою очередь способствует субадаптивным и неадаптивным реакциям организма, усугубляющим проблему. Пребывание в состоянии дистресса может отражаться на состоянии здоровья в целом и на реализации репродуктивной функции в частности. В обзоре обсуждаются литературные сведения, касающиеся влияния стресса матери на фолликулогенез, в том числе на фоне применения вспомогательных репродуктивных технологий (ВРТ). Сопоставлены клинические данные и результаты экспериментальных исследований на лабораторных животных о влиянии психоэмоционального стресса до и во время беременности на созревание ооцитов и развитие эмбрионов. Особое внимание уделено влиянию

психоэмоционального стресса на репродуктивную функцию на фоне применения ВРТ, когда введение экзогенных гормонов компенсирует вызванное стрессом нарушение функции гипоталамо-гипофизарно-гонадной оси. При применении ВРТ стресс-индуцированное нарушение оогенеза реализуется не через путь снижения выработки гормонов гипоталамуса и гипофиза, а через другие механизмы, в частности паракринные, которые описаны в этом обзоре. В результате проведенного анализа литературы можно сделать заключение о том, что стресс негативно влияет на развитие ооцитов яичника, а также последующее развитие полученных из них эмбрионов, выявлена роль некоторых паракринных факторов яичника, таких как BDNF, GDF-9, HB-EGF, TNF-α и др., которые задействованы в этих процессах.
Ключевые слова: стресс; отдаленные эффекты; фолликулогенез; вспомогательные репродуктивные технологии; преимплантационный эмбрион.

Introduction

Adult reproductive function and children's health are in focus of scientific interest and of a great public concern. The implementation of assisted reproductive technologies (ARTs) into clinical practice helps to overcome many types of infertility, miscarriage, and to prevent monogenic diseases in children. At the same time, patients in ART clinics often report that the infertility itself as well as its treatment is the traumatic experience, which may lead to the anxiety and even depression (Cousineau, Domar, 2007; Rockliff et al., 2014). Thus, chronic psycho-emotional stress that affects both women and men during infertility treatment and implementation of the ART in particular, are significant factors affecting fertility.

There are numeric evidences of the negative impact of chronic stress on the human well-being, on the mammalian physiology in general, and on the reproductive function in particular (Louis et al., 2011; Muscatell, Eisenberger, 2012). The effects of maternal stress during pregnancy on the body weight of newborns and on the neurodevelopment in children are reported; moreover, there are evidences that prenatal stress affects the behavior and other phenotypic characteristics of different animals (Weinstock, 2008, 2016; Ragaeva et al., 2018; Fitzgerald et al., 2021).

Although the effects of psycho-emotional stress are described in the medical literature and on the laboratory animal models, these two areas of research are mostly developing independently. It should be noted, that nowadays ART is widely used in medical practice, thus the effects of psycho-emotional stress on reproduction, including stress arising from the use of ART, as well as studying the mechanisms underlying these effects are of great concern. The objective of this article is to review and to systematize the accumulated experimental and clinical data describing the effects of chronic psycho-emotional stress on gametogenesis, fertility, ART outcomes, and the offspring health; to review animal models used in such experiments and to outline possible ways aiming to mitigate the adverse effects of stress associated with the use of ARTs. At the same time, experimental data obtained on animal models are compared with clinical observations published in the medical literature. The effects of stress on folliculogenesis and embryogenesis, as well as on the ART-born offspring, both in humans and in experimental animals, are analyzed.

Modeling psycho-emotional stress in laboratory animals

In experimental studies aimed to elucidate the effects of chronic psycho-emotional stress, including the stress associated with the use of ARTs, on the development of oocytes and early embryos, animal model of restriction stress (Burkus et al., 2013; Gao et al., 2016), the predator exposure model (Liu et al., 2012; Di Natale et al., 2019), or the model of chronic unpredictable mild stress – CUMS (Wu L.M. et al., 2012a, b; Gao et al., 2016) are most frequently used. Plasma levels of corticosteroids, adrenocorticotropic hormone, corticotropin-releasing hormone, adrenaline, noradrenaline, and ghrelin are normally measured in such studies as stress indicators; less often stress-induced analgesia, behavioral characteristics are also taken into account.

The restriction model of stress is one of the most popular (Gao et al., 2016). Sometimes the experimental animal is fixed with tapes, plaster, cloth towel, or other means so that only the head can move freely; however, most often for this purpose animal is settled in a plastic or metal tube, or a special microcell restricting its movements (Zhang et al., 2011; Gao et al., 2016). The duration of the procedure and the number of restriction episodes affect the intensity of the stress response and should be taken into account (Zhang et al., 2011; Wu X.F. et al., 2015; Zhao X.Y. et al., 2020).

Predator model of psychogenic stress is also widely used, for this purpose the natural predators of mice such as cats, ferrets, rats or foxes are normally chosen; sometimes not the predator itself, but its smell is offered to the tested mouse, this causes the fear and anxiety in the experimental animal (Sanchez-Gonzalez et al., 2018; Di Natale et al., 2019). The most commonly used version of the predator stress model for mice is the presentation of a hungry cat or its scent without physical contact between the mouse and the cat (Liu et al., 2012). The presence of the cat affects the stressed animal, and activating its hypothalamic-pituitary-adrenal axis, therefore triggering the secretion of glucocorticoids (Sanchez-Gonzalez et al., 2018).

Another widely used model is CUMS. Rodents are presented with constantly changing variable stressors over several weeks (Campos et al., 2013). According to this model, combination of isolation and overcrowding can be used as stressors, as well as unpredictable changing the situation

in the cage: wet sawdust, tilting the cage, disruption of the day-night cycle, exposure to different temperatures, the use of mobility restrictions, social stress (Haller et al., 1999; Gao et al., 2016; Burstein, Doron, 2018; Gadek-Michalska et al., 2019). After several days of CUMS regimen, animals show an increase in blood corticosterone levels and a reduced response to pleasurable stimuli (Campos et al., 2013; Gadek-Michalska et al., 2019).

Influence of stress on the reproductive function of mammals: experimental data

Animal studies demonstrate that psycho-emotional stress experienced by the female affects the quantity and quality of oocytes, which in turn contributes to further embryonic development (see the Table). Many studies come to the conclusion that stress leads to a decrease in the developmental potential of oocytes (Wiebold et al., 1986; Zhang et al., 2011; Liu et al., 2012; Lian et al., 2013; Wu X.F. et al., 2015; Gao et al., 2016); this, in turn, resulted in a reduced percentage of blastocysts developed from such oocytes. A decrease in the developmental potential of oocytes was associated with the duration and severity of the applied stress treatment (Gao et al., 2016). It was also revealed, that antral follicles are more sensitive to stress than preantral ones (Gao et al., 2016). Moreover, chronic unpredictable stress disrupts ovulation and cyclicity in female mice, these changes in reproductive system correlate with high levels of corticosteroids in the blood and with the increased activity of superoxide dismutase; moreover, after hormonally induced stimulation of superovulation, mature oocytes were not found in the stressed female mice (Kala, Nivsarkar, 2016).

Stress can also affect embryo implantation. It was shown that even a short restriction stress lasting 24 hours, but coinciding in time with the "implantation window" on the fourth day after mating, negatively affects implantation in mice and slows down the onset of hatching in blastocysts (Zhao L.H. et al., 2013). This effect was mediated through a decrease in the blood levels of progesterone and estradiol, and was associated with the level of expression of heparin-binding epidermal growth factor both in the uterus and in the blastocysts (Zhao L.H. et al., 2013).

It is known that stress leads to activation of the hypothalamic-pituitary-adrenal and sympathoadrenal systems; therefore, traditional markers of stress are glucocorticoids and adrenaline. Restriction stress in mice was shown to be accompanied by an increase in plasma cortisol levels (Zhang et al., 2011). Cortisol injections also led to suppression in oocyte development. In addition, stress led to a decrease in the follicle-stimulating hormone (FSH) release, while injections of cortisol did not cause this effect. The researchers concluded that cortisol affects oocytes through a direct effect on the ovary, while stress impairs their competence indirectly, via effects on the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-ovarian axes (Zhang et al., 2011).

One of the ways to implement stressful effects on the female reproductive system is to influence the production of ovarian regulators of folliculogenesis, including those mediated by corticotropin-releasing hormone. Corticotropin-releasing hormone (CRH), which is identified in the theca and stroma of the ovaries, as well as in the cytoplasm of oocytes and granulosa cells, is involved in the regulation of follicular maturation, ovulation, the formation of *corpus luteum*, and the synthesis of the ovarian steroid hormones (Kiapekou et al., 2010; Zhai et al., 2020).

In female mice, restriction stress caused an increase of CRH concentration in blood serum, ovaries, and oocytes, as well as an increase in the expression of the CRH receptor 1 (CRHR1) in granulosa and theca cells, but a decrease in the expression of the glucocorticoid receptor and brain-derived neurotrophic factor (BDNF) in the ovaries (Liang et al., 2013). All this ultimately led to an imbalance between estradiol and progesterone concentration in blood and negatively affected the developmental competence of oocytes. Besides, the addition of CRH to the culture medium during oocyte's *in vitro* maturation disrupted its development and increased the rate of apoptosis in granulosa cells (Liang et al., 2013). In another study, it was shown that a stress-induced increase of CRH both in blood and in the ovaries of female mice triggers apoptosis in oocytes and in ovarian granulosa cells due to the activation of the TNF-α system, which results in impaired oocyte competence (Zhao X.Y. et al., 2020).

Animal experiments using CUMS stress model demonstrated that inhibition of follicle development is associated not only with gonadotropins, but also with growth factors such as growth differentiation factor 9 (GDF-9) and BDNF (Wu L.M. et al., 2012b). Exposure of female mice to CUMS resulted in the suppressed follicular development, increased level of follicular atresia, and downregulated GDF-9 expression. The introduction of exogenous gonadotropins partially mitigated these negative effects and restored the development of antral follicles, which was suppressed due to chronic stress, but these exogenous gonadotropins exerted no effects on secondary follicles. However, the introduction of recombinant GDF-9 restored the development of secondary follicles. Co-administration of GDF-9 and gonadotropins in stressed mice restored both secondary and antral follicles. Another study of the same research group showed that CUMS reduces BDNF expression in antral follicles but does not affect BDNF expression in primordial, primary, and secondary follicles (Wu L.M. et al., 2012a). Chronic unpredictable mild stress also reduced the number of retrieved oocytes and the percentage of blastocysts formed, which was corrected by the use of exogenous BDNF.

Some studies attempt to elucidate mechanisms of the influence of psycho-emotional stress on the developmental potential of oocytes and preimplantation embryos. One study reported, that the transition of the heterochromatin configuration from the non-surrounded nucleolus (NSN) type to the surrounded nucleolus (SN) type is suppressed at the germinal vesicle (GV) stage preovulatory oocytes exposed to restriction stress, thus, the developmental potential of such oocytes is impaired (Wu X.F. et al., 2015).

Effects of stress exposures on the development of oocytes and embryos in mice

Stress model	Time of stress exposures	The effect of stress	Reference
The presence of a predator	Within 24 h after the administration of the equine chorionic gonadotropin (eCG)	Impaired *in vivo* and *in vitro* development of oocytes and embryos	Liu et al., 2012
Restriction	Daily for 8 h for 4, or 8, or 15, or 23 days immediately prior to the administration of eCG to female mice, i. e. 48 h before collecting early embryos	Decreased *in vitro* development of embryos collected from mice subjected to stress during 8 or more days	Gao et al., 2016
	Within 24 h, on the 4th day of pregnancy in mice, i. e. during "implantation window"	Decreased number of implantation sites. Slowing down of blastocyst hatching. A decrease in the concentration of progesterone and estradiol in the blood and a decrease in the expression of HB-EGF* in the endometrium of the uterus and in the blastocysts	Zhao L.H. et al., 2013
	Within 48 h, immediately prior to the onset of pregnancy	Increased corticotropin-releasing hormone in the blood and ovaries. Activation of the TNF-α** system. Triggering of apoptosis in oocytes and ovarian granulosa cells. Disruption of the competence of oocytes	Zhao X.Y. et al., 2020
	24 h, 48 h or 23 days prior to hormonal stimulation and oocyte retrieval	Both 48 h and 23 days of restriction were accompanied by a decrease in histone acetylation and methylation, which ultimately reduced the oocyte development	Wu X.F. et al., 2015
	24 h or 16 h before oocyte retrieval	An increase in the level of corticotropin-releasing hormone in the blood serum, ovaries and oocytes. Increased apoptosis in cumulus cells, leading to a disruption of the oocyte development	Liang et al., 2013
		Disruption of the spindle apparatus at the MI stage. An increase in the proportion of aneuploidy in mature oocytes	Zhou et al., 2012
		Accumulation of ROS in blood serum, ovaries and oocytes. Decrease in the percentage of developing blastocysts and the low cell number in these blastocysts	Lian et al., 2013
	Within 24 h and 48 h during the period of growth and maturation of oocytes (proestrus in unstimulated mice (24 h), early (0–24 h) and late (24–48 h) stages of oocyte development in hormonally stimulated mice)	Disorder ovulation, decreased percentage of developed blastocysts and the number of cells in them, and fewer live offspring born after the embryo transfer to recipients compared with unstressed controls	Zhang et al., 2011
Chronic unpredictable mild stress (CUMS)	Exposure to various stressors: hot air, swimming in water of different temperatures, shaking (daily, for 4 days, twice a day)	Increase in the percentage of atretic antral follicles; a reduced percentage of 4-cell embryos and blastocysts, fewer cells in blastocysts, and fewer live offspring born after the embryo transfer	Gao et al., 2016
	For five days once a day (30–60 min) exposure to various stressors: restriction; being in a slanted cage or in a cage with dirty bedding; isolation; lack of bedding	Estrous cycle disorders. An increased percentage of atretic antral follicles. Anovulation. The number of oocytes retrieved from stressed mice after hormonal ovarian stimulation was reduced and there were no mature oocyte stages	Kala, Nivsarkar, 2016
	For 30 days, different stressors: isolation; overcrowding and cage tilt; swimming in cold water; hot air; lack of food and water; wet bedding; shaking; shift of the light-dark period	Suppression of development and atresia of follicles. Suppression of GDF-9*** expression	Wu L.M. et al., 2012b
		Decreased expression of BDNF**** in antral follicles. No effect on the BDNF expression in primordial, primary and secondary follicles. Reducing the number of oocytes retrieved and the percentage of blastocysts developed	Wu L.M. et al., 2012a

* HB-EGF, heparin-binding EGF-like growth factor.
** TNF-α, tumor necrosis factor alpha.
*** GDF-9, growth differentiation factor 9.
**** BDNF, brain-derived neurotrophic factor.

Besides, psycho-emotional stress can lead to disruption of meiotic division in oocytes. It was shown that in stressed females, the proportion of aneuploidy in mature oocytes increases, and the percentage of aneuploid oocytes was three times higher in oocytes with accelerated maturation compared to the delayed ones (Zhou et al., 2012).The authors concluded that maternal stress may cause oxidative stress within oocytes and impair spindle assembly by inactivating the spindle-assembly checkpoint (Zhou et al., 2012).

In addition to hormonal imbalance, psychosocial stress causes an increase in the formation of reactive oxygen species (ROS). High levels of ROS cause oxidative stress, which leads to meiotic cell cycle arrest and resulted in apoptosis (Prasad et al., 2016; Chaudhary et al., 2019). This conclusion was further supported by the observation that oxidative stress induces granulosa cell apoptosis and leads to a decrease in estradiol levels, ovulation frequency, and oocyte quality (Tripathi et al., 2013). Besides, oxidative stress-induced apoptosis of granulosa cells caused the impairments of the contacts of these cells with oocytes, which directly affects the supply of nutrients and the availability of growth factors that affect the quality of oocytes in preovulatory ovarian follicles (Prasad et al., 2016). In experiments using the restriction stress model, it was shown that stress caused the accumulation of ROS in the blood serum of mice, ovaries, and oocytes, and also caused a decrease in the percentage of blastocysts developing *in vitro* with fewer cells observed in these blastocysts (Lian et al., 2013).

It should be noted that the mechanism of the negative impact of chronic stress on the ovary through inhibition of the release of gonadotropins has been well studied. While other ovarian regulatory mechanisms involved in this process are not yet understood. Elucidating these paracrine mechanisms mediating the effects of stress on oogenesis and, subsequently, on the development of embryos is important for more effective use of medical reproductive technologies in patients experiencing chronic psycho-emotional stress.

Impact of stress on female reproductive function: clinical data

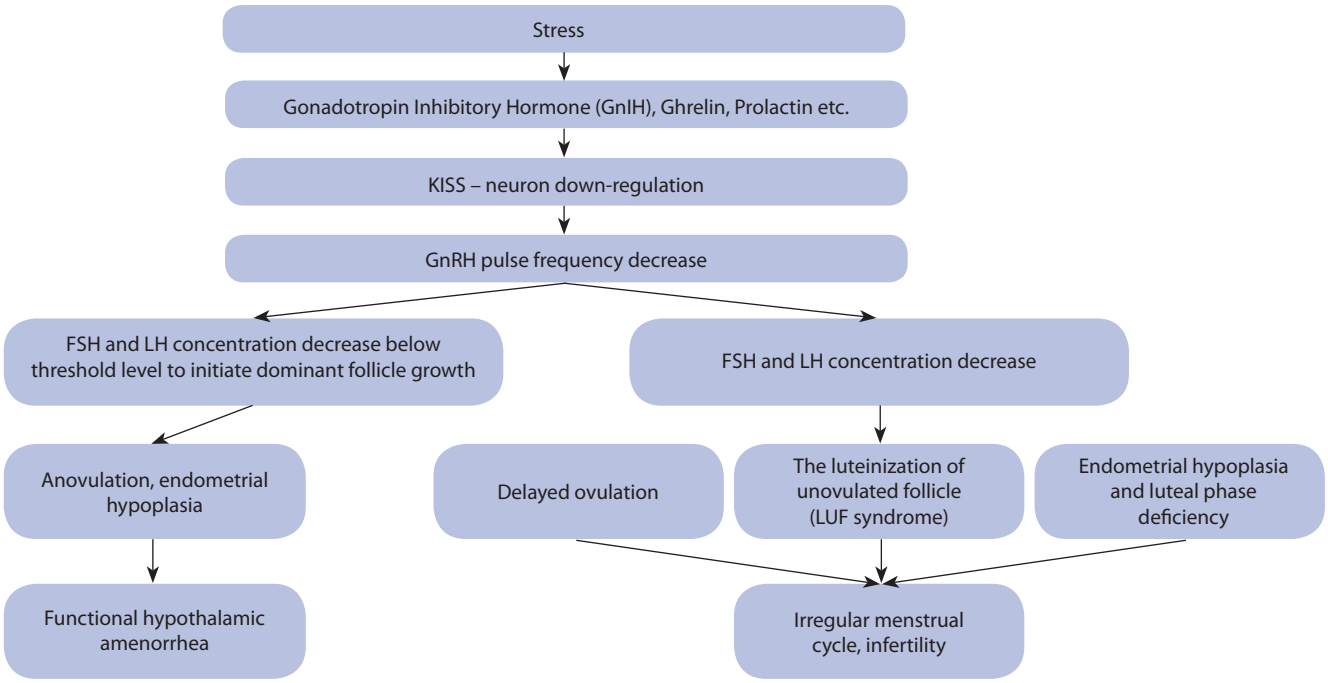
Clinical data without ART

Functional hypothalamic amenorrhea which may be diagnosed in the absence of menstruation for three or more months represents the most striking clinical manifestation of stress-induced disorders of folliculogenesis, with initially intact menstrual function (Warren, Fried, 2001). First of all, functional hypothalamic amenorrhea is characterized by a decrease in the frequency and amplitude of gonadotropin-releasing hormone release peaks, what leads to a decrease in the production of FSH and luteinizing hormone (LH) by the pituitary gland and, as a result, to the absence of hormone-dependent follicle growth. This leads to a disruption of the transition from secondary follicles to antral follicles, a disruption of the formation of a pool of growing follicles, the absence of a dominant follicle and, accordingly, lack of the corpus luteum. Therefore, functional hypothalamic amenorrhea is characterized by a decrease in the production of estradiol in the ovaries, which is accompanied by the absence of uterine endometrial proliferation and the absence of menstruation (Fourman, Fazeli, 2015; Prokai, Berga, 2016).

Manifestations of other disorders of folliculogenesis caused by stress may not be as pronounced as amenorrhea. Subclinical manifestations include lengthening and irregularity of the menstrual cycle, insufficiency of the luteal phase of the cycle, luteinization of the non-ovulated follicles (Berga, Loucks, 2007; Palm-Fischbacher, Ehlert, 2014), as well as the absence of a mature oocytes in the ovulated follicle (Tamura et al., 2013). In the case of subclinical manifestations, there is also a decrease in the production of gonadotropin-releasing hormone, however, FSH still reaches a level sufficient to initiate follicle growth. In this case, the growth of the follicles may be slow, with the prolongation of the first phase of the cycle, the late ovulation, and the reduced production of estradiol, which can lead to endometrial hypoplasia (Berga, Loucks, 2007; McEwen et al., 2012). Changes in gonadotropin-releasing hormone pulsation also affect the intensity and frequency of LH peaks, although serum LH levels may remain normal or be only slightly reduced (Krsmanovic et al., 2009). Deficiency in the production of gonadotropin-releasing hormone and estradiol can lead to a reduced LH peak in the preovulatory period and to luteinization of the unovulated follicle, the formation of ovarian cysts, luteal phase insufficiency, and the reduced progesterone production in the second phase of the cycle (Berga, Loucks, 2007). Besides, chronic stress is characterized by increased secretion of cortisol mainly at night, with normal levels of daily and morning secretion (McEwen, 2000), which also contributes to a decrease in the amplitude or even the absence of the ovulatory peak of gonadotropins (Cahill et al., 1998).

Certainly, not all women demonstrate disruption of folliculogenesis and the distortion of the menstrual cycle in the stressful situations (McComb et al., 2006; Ellison et al., 2007). There are both physiological and psychological factors determining tolerance to stress-induced ovarian dysfunction (Wingfield, Sapolsky, 2003; Palm-Fischbacher, Ehlert, 2014). Factors that protect the reproductive system in the situation of stress operate at the level of the central nervous system (the stressor is not perceived), at the level of the hypothalamic-pituitary-adrenal system (impaired secretion of glucocorticoids), at the level of the hypothalamic-pituitary-gonadal system (resistance of the gonads to the action of glucocorticoids), and protection from the action of glucocorticosteroids with the help of a proteins that bind steroids (Wingfield, Sapolsky, 2003).

The development of hypothalamic amenorrhea leads to the reproductive failure due to the absence of follicular growth and the lack of matured oocytes, as well as the lack of appropriate preparation of the endometrium. Sometimes in the case of stressful influences with the formation of luteinization of the unovulated follicle and a deficiency of the luteal phase, the absence of menstruation (amenorrhea) is not observed, but the reproductive potential is significantly reduced (Lynch et al., 2014). Such alterations can be cor-



Effects of stress on female reproductive function.

rected by the proscription of appropriate medical treatment that compensate the deficiency of pituitary and/or steroid hormones. However, there is the problem of infertility of unknown origin, with unimpaired folliculogenesis and ovulation. A more detailed study of oogenesis became possible with the introduction of ART.

There are several evidences of a high rate of early pregnancy loss, not associated with chromosomal abnormalities, or an increased anxiety and depression in women with a history of miscarriages. Pregnancy loss itself may be considered as a powerful stressor, which can lead to the recurrent miscarriage (Quenby et al., 2021; Wang et al., 2021). Early pregnancy loss indicates a low viability of the embryo or lack of its interaction with the uterus after implantation, which is probably the result of gametogenesis disruption, distortion in preparation of the endometrium for pregnancy or the development of immunological incompatibility between the maternal organism and the embryo. All of these conditions have been described as possible consequences of stress experienced by a woman during conception and early pregnancy (Wadhwa, 2005; Nepomnaschy et al., 2006). These phenomena can lead to impaired placentation and the development of pregnancy complications typical for later stages of gestation, such as fetoplacental insufficiency, preeclampsia, preterm birth; these conditions may affect the health of children born (Parker, Douglas, 2010; Witt et al., 2012). Mechanisms which cause a change in ovarion function under stress are shown in the Figure.

Clinical data obtained with the use of ART

The use of gonadotropins to induce the growth of several follicles for controlled ovarian hyperstimulation is one of the core ARTs. Thus the stress-induced deficiency of CRH, FSH, and LH, which was discussed in the previous section, is compensated by the administration of exogenous gonadotropins. Moreover, current protocols for controlled ovarian hyperstimulation involve blockade of endogenous gonadotropin-releasing hormone production in order to prevent premature ovulation. Taking all this into account, it can be concluded that during the use of ART, stress-induced disturbance of oogenesis is realized not through a decrease in the production of hormones of the hypothalamus and the pituitary gland, but via other paracrine and autocrine mechanisms described above.

Many reproductologists noticed that unsuccessful ART attempts pretty often take place during adverse life events, such as death of a relative, family problems, etc. This is in practice described by reproductologists as cases of "inexplicably low" quality/quantity of oocytes and embryos in ART programs with an initially good prognosis, and "unexplained" improvement in the quality/quantity of oocytes and embryos in repeated attempts using the same protocols when life situation of the patient was improved (Ebbesen et al., 2009; Meldrum, 2016). A possible explanation for such observations is the inhibitory effect of the stress on gametogenesis.

Data from clinical studies in humans are contradictory. A significant part of these studies indicates the depressing effect of psychological stress on the results of ART. Thus, in a study by Ebessen et al. (2009) involving 809 women practicing ART for the first time, a decrease in the number of received oocytes, embryo quality and pregnancy rate was shown with an increase in the number of adverse life events that reduce the quality of life, as well as with an increase in the level of perceived stress one month before infertility

treatment with ART (Ebbesen et al., 2009). Another study showed the effect of initial stress on the number of oocytes retrieved and fertilized, as well as on pregnancy and live birth rates (Klonoff-Cohen et al., 2001). Li et al. (2011) found that initial psychological stress was negatively associated with pregnancy rates in ART programs, but intrafollicular concentrations of norepinephrine did not differ between the pregnant and non-pregnant women. In another study, the association of ART results with anxiety levels and serum concentrations of cortisol and noradrenaline was investigated. Elevated levels of cortisol and norepinephrine have been shown to be associated with anxiety levels and negatively correlated with pregnancy and live birth rates (An et al., 2013).

A more recent paper published the results of a study with 135 women involved, that examined the association of salivary and hair cortisol with ART outcomes (Massey et al., 2016). It was shown that salivary cortisol levels were not predictive of ART outcomes. Whereas, lower hair cortisol concentrations predicted the high probability of pregnancy. A recent study of 304 women found that more than 80 % of respondents had elevated levels of anxiety and depression, and these symptoms were inversely correlated with the success of ART implementation (Aimagambetova et al., 2020).

At the same time, some studies do not show an association between anxiety levels, as well as salivary and serum cortisol levels and reproductive outcomes in ART patients (Lovely et al., 2003; Cesta et al., 2018). Miller et al. (2019) assessed the level of anxiety using the Perceived Stress Scale, salivary cortisol concentration at the beginning of the ART cycle, on the day of follicle puncture, and on the day of embryo transfer, and also measured the level of cortisol in the follicular fluid. The authors noted an increase in cortisol and anxiety on the day of follicle puncture, but did not find an association of these stress indicators with pregnancy rates. Besides, elevated follicular cortisol levels correlated with positive ART outcomes. *In vitro* fertilization (IVF) failure has also been shown to predict subsequent psychological distress, but pre-IVF psychological distress did not predict IVF failure (Pasch et al., 2012). The level of stress and the number of oocytes obtained in ART programs for the treatment of infertility was compared. The results of this study showed a significantly higher level of stress in patients with infertility, but the number of oocytes was comparable in both groups (Adeleye et al., 2020).

It can be concluded that despite the large number of publications addressing the effects of stress on the effectiveness of ART in humans, the data obtained are very contradictory. In these studies not only sizes of study groups are variable, but also different approaches to assess experienced stress and anxiety were used. Moreover, many of these studies suffer from the lack of randomization. The majority of these studies do not take into account the fact that the patient's knowledge of his prognosis can significantly affect the assessment of the level of chronic stress and the results of the questionnaire. Due to the heterogeneity of the published data, the conclusions of these works are also contradictory. Most authors are careful in conclusions about the relationship between stress and reproductive function, based both on the data of their studies and on the general biological considerations suggesting the impossibility of complete suppression of the reproductive function during unfavorable periods due to the need for the survival of the species (Wingfield, Sapolsky, 2003; Rooney, Domar, 2018; Lawson, 2020).

Psychotherapy as a way to mitigate the negative effect of psycho-emotional stress on the reproductive system

The availability of data indicating a significant impact of psychosocial stress on the reproductive system has contributed to an increase in research aimed at studying psychotherapeutic effects in the treatment of infertility.

An early paper addressing this issue highlights the urgent need for quality-compliant research feasible for evaluation (Boivin, 2003). The author analyzed 38 studies, 25 of which were classified as independent, and only eight of them met the research quality standards. In summary, three out of eight good quality studies showed higher pregnancy rates in the psychosocial intervention group compared to the routine care group (Boivin, 2003). In another paper, a meta-analysis of 22 studies was conducted, which indicates that psychotherapy (group and individual/couple) reduced anxiety and depression in infertile patients and possibly affected the success rate of conception (Liz, Strauss, 2005). A review by Campagne (2006) recommends planning of infertility medication taking into account the level of stress, and suggests stress-reducing therapies, prior to initiating infertility treatment (Campagne, 2006).

Subsequent studies presented conflicting results of the use of psychological techniques. Hämmerli et al. (2009) included 21 controlled trials in their meta-analysis and concluded that psychological interventions were not associated with any significant changes in psychological status, but had a positive effect on pregnancy rates in patients receiving treatment without ART (Hämmerli et al., 2009). They also concluded that a therapy of six or more sessions was more effective than a shorter duration of therapy. Frederiksen et al. (2015) performed a meta-analysis of 39 original articles and reported that women receiving some form of psychotherapeutic intervention were about twice as likely to become pregnant compared with women receiving standard treatment (Frederiksen et al., 2015).

Ying et al. (2016) included 20 randomized trials in their systematic review. They concluded that there were methodological problems with studies that reported significant effects of psychological stress on the pregnancy rates, and recommended that a more thorough investigation to be conducted, especially for the most stressful period for infertile patients, in particular, during the time of waiting for the results of a pregnancy test. In a systematic review by Gaitzsch et al. (2020), only two of six studies showed a significant positive effect of psychological interventions on the fertility (Gaitzsch et al., 2020). At the same time, a meta-analysis including 15 studies showed a positive

association between psychosocial interventions, especially long-term ones, and pregnancy rates in infertile women and couples receiving ART treatment (Katyala et al., 2021).

Thus, many researchers emphasize the presence of methodological and practical questions to the currently accumulated data. There is a need for more studies and for unified programs of psychological help. The positive effects of psychotherapy demonstrated in some of the studies indicate that this is a promising area for further research.

Conclusion

The identification of chronic psycho-emotional stress is challenging both in humans and in experimental studies with laboratory animals. The psychological tests and questionnaires in humans are considered as the "gold standard" for such psycho-emotional stress identifying, however, it requires a lot of time, may not reflect the real physiological situation due to subjective distortions introduced by the interviewee (Slavich, Shields, 2018; Crosswell, Lockwood, 2020). It is also important that the use of psychological tests and questionnaires is not possible in experiments with laboratory animals. Therefore, the search for reliable biomarkers of chronic psycho-emotional stress which can be objectively measured and evaluated is extremely important.

The use of animal models helps to understand the mechanisms underlying the impact of assisted reproductive technology accompanied by stress on the female reproductive function and on the offspring health. Analysis of the literature let to conclude that stress negatively affects the development of ovarian oocytes, as well as the subsequent embryo development. The role of some ovarian paracrine factors that are involved in these processes has been revealed in these studies. Meanwhile, additional experiments on the effect of psycho-emotional stress on the results of *in vitro* fertilization and embryo transfer experiments are warranted, since clinical data are contradictory and only a few experimental works on laboratory animals are available so far.

Available data on the laboratory animals show the effectiveness for the use of such factors as GDF-9 and BDNF to reduce the inhibitory effect of stress on folliculogenesis and embryo development, these factors are promising to be used in the reproductive medicine. Moreover, psychotherapeutic techniques which alleviate effects of stress may increase resistance to stress at the level of the central nervous system, i. e. influence the perception of a stressful event or stimulus. There are reports confirming the effectiveness of psychological techniques in reducing psychological stress, and there are evidences that the use of these techniques is associated with a significant increase in pregnancy rates (Hämmerli et al., 2009; Frederiksen et al., 2015; Katyala et al., 2021). It is important to increase the availability of psychotherapy in reproductive medicine, especially taking into account the level of stress reported by infertile women.

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Aspects of the rhizospheric microbiota and their interactions with the soil ecosystem

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Abstract. Soil microbial communities play a key role in the evolution of the rhizosphere. In addition, proper exploration of these microbial resources represents a promising strategy that guarantees the health and sustainability of all ecosystems connected to the ground. Under the influence of environmental conditions, microbial communities can change compositions in terms of abundance and diversity. Beyond the descriptive level, the current orientation of microbial ecology is to link these structures to the functioning of ecosystems; specifically, to understand the effect of environmental factors on the functional structure of microbial communities in ecosystems. This review focuses on the main interactions between the indigenous soil microflora and the major constituents of the rhizosphere to understand, on the one hand, how microbial biodiversity can improve plant growth and maintain homeostasis of the rhizospheric ecosystem, on the other hand, how the maintenance and enrichment of plant biodiversity can contribute to the conservation of soil microbial diversity; knowing that these microorganisms are also controlled by the abiotic properties of the soil. Overall, understanding the dynamics of the rhizosphere microbiome is essential for developing innovative strategies in the field of protecting and maintaining the proper functioning of the soil ecosystem.

Key words: soil microorganisms; rhizosphere; microbial diversity; plant biodiversity.

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Аспекты ризосферной микробиоты и их взаимодействие с почвенной экосистемой

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Аннотация. Почвенные микробные сообщества играют ключевую роль в эволюции ризосферы. Планомерное изучение этих микробных ресурсов представляет собой перспективную стратегию, с помощью которой можно будет обеспечить здоровье и устойчивость всех почвенных экосистем. Под воздействием окружающей среды микробные сообщества могут менять численность своих популяций и видовой состав. Современная микробная экология нацелена, помимо описательного уровня, на определение связей этих структур с функционированием экосистем, в частности для понимания роли окружающей среды в жизнедеятельности микробных сообществ в экосистемах. Настоящий обзор посвящен основным взаимодействиям между местной почвенной микрофлорой и главными составляющими ризосферы. Важно понять, с одной стороны, , как микробное биоразнообразие может улучшить рост растений и поддержать гомеостаз ризосферной экосистемы, а с другой – как сохранение и повышение растительного биоразнообразия способствуют сохранению почвенного микробного разнообразия, зная при этом, что данные микроорганизмы контролируются еще и абиотическими свойствами почв. В целом понимание динамики микробиома ризосферы необходимо для разработки инновационных стратегий в области защиты и поддержания надежного функционирования почвенной экосистемы.

Ключевые слова: почвенные микроорганизмы; ризосфера; микробное разнообразие; биоразнообразие растений.

Introduction

The rhizosphere, a narrow area of soil that surrounds the roots of plants, harbors a number of microorganisms that interact with plants and the surrounding soil, and is considered one of the most dynamic interfaces on Earth (Philippot et al., 2013; Kuzyakov, Blagodatskaya, 2015). In addition, since their colonization of terrestrial environments, terrestrial plants have formed symbioses with microorganisms (Fitzpatrick et al., 2018). They have been accompanied by fungi, bacteria, viruses and protists over millions of years, and those associations that allow and accelerate the adaptation of plants to life on Earth (Shekhar et al., 2019).

It has been estimated that the symbiosis between plants and fungi was established early with arbuscular mycorrhizal fungi more than 450 million years ago (Ma) during the colonization of the Earth by plants, as the oldest and the most common symbiotic association of plants with microbes (Field et al., 2015). However, the structure and activity of soil microbial communities are intimately linked to their roles in ecological processes; the identity and abundance of species present in an ecosystem determine the types of interactions in the rhizosphere and subsequently constitute the key elements of the ecological theories (Talbot et al., 2014). In addition, the soil microbiome is divided into two distinct microbial compartments, depending on their position in relation to the roots of plants, the microorganisms surrounding the roots being commonly referred to as rhizospheric or endophytes (Fitzpatrick et al., 2018).

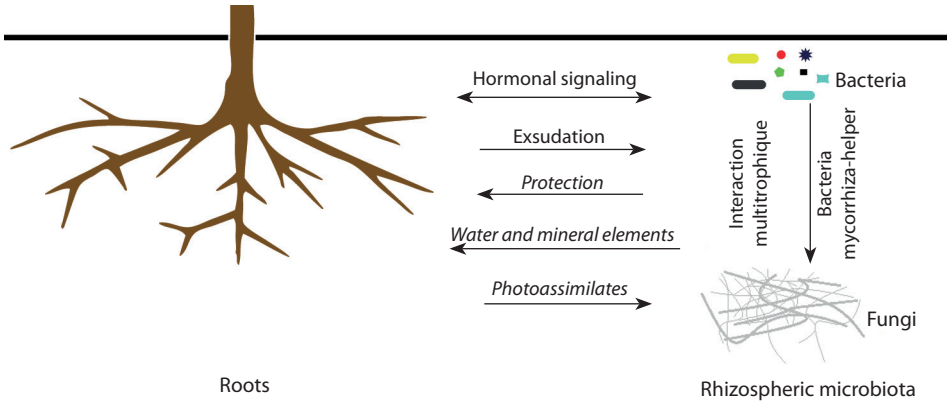
Interactions between the plant and its microbiota range from parasitism to mutualism, and their results can be decisive for the performance of the plant (Almario et al., 2017; El Amrani, Amraoui, 2022). Endophytic soil microorganisms colonize plant roots forming complex communities and perform beneficial functions by improving plant growth, health and defense against enemies. This association improves the adaptation of plants to environmental constraints such as drought and nutrient deficiency (Almario et al., 2017; Shekhar et al., 2019). This beneficial effect of the root microbiota on plants is achieved by the secretion of different growth hormones such as auxin, cytokinin and gibberellic acid, or by reducing the production of ethylene. This leads to the promotion of plant growth by changing the architecture of the root system (Shekhar et al., 2019; El Amrani, Amraoui, 2020) and also by increasing the acquisition of nutrients (Fitzpatrick et al., 2018).

Thus, the plant microbiota can be considered as an extension of the plant, in the sense that it can increase the plant's access to nutrients in poor soils (Vandenkoornhuyse et al., 2015). It has been estimated that 80 % of vascular plant species receive phosphorus (P) and other nutrients from fungi in exchange for photosynthesis (Almario et al., 2017) (see the Figure). In other words, microbial biodiversity is essential to enhance the sustainable growth of plants through improved nutrition, root architecture, defense mechanisms and the competition with pathogens as well as through participation in the adaptation of plants to abiotic constraints.

The concept of microbial biodiversity

Bacteria are the most diverse organisms among living things (Whitman et al., 1998). Their activity, richness and composition play a major role in the functioning of an ecosystem, either free-living or associated with other host organisms (Walters, Martiny, 2020).

Microbial biodiversity studies use several methods depending on the objective. Species diversity is the most studied concept despite it being a single dimension of biodiversity (Latimer, 2012). This is due to the fact that species is the basic unit of ecology and the evolution of ecosystems, hence the importance of this concept for evaluating and conserving biodiversity. However, definitions and formulas have been developed to fully understand and control microbial communities (Fontana et al., 2020). These notions include the measurement of biodiversity at spatial scales; according to Whittaker (1972), this notion is based on three scopes: (i) alpha diversity refers to the diversity within a particular ecosystem (number or relative abundance of taxa); (ii) beta diversity expresses the total number of species unique to each of the ecosystems compared; it makes it possible to examine the evolution of the diversity of species between several ecosystems; (iii) the total or gamma diversity of a landscape, or geographical area, is the product of the alpha diversity of its communities and the degree of beta differentiation between them. Among these three parameters, alpha diversity is the key element in conservation work because it quantifies the biodiversity of a particular ecosystem through measurement



Schematic of the interactions between roots and soil microbial communities.

based on the notion of presence/absence and abundance of taxa within a local community.

Despite the diversification of these mathematical tools, they fail to reflect the added value of diversity within the ecological whole. In this regard, the notion of functional diversity versus specific diversity appeared (Biswas, Mallik, 2011). This functional diversity is based on a metric for quantifying the diversity of functional traits (Díaz et al., 2007). This calls into question the philosophy of conservation biology, which recognizes that the great diversity of species ensures great functional diversity and maintains the functional stability of the ecosystem (Mayfield et al., 2010).

Factors and interactions of soil microorganisms

Soil microbial communities are vital for an ecosystem to maintain the sustainability of long-term ecological interactions (Chang et al., 2017). They are essential to the plant due to their contribution to its growth, its development and its productivity (Trivedi et al., 2013) through the maintenance of soil fertility thanks to the important roles they play in the availability of nutrients (Chang et al., 2017). Soil microbial communities also play a fundamental role in soil biogeochemical cycles (Rousk, Bengtson, 2014) because the dynamic structure of soil largely depends on the interaction between microbial biology and the roots of plants living in the soil (Jin K. et al., 2013).

However, there are a variety of factors that can significantly affect soil microbial communities and predict the abundance and diversity of these communities. Among these factors, there are biotic factors such as root respiration and the nature of forest formation (Chen et al., 2015b; Schmid et al., 2019); and abiotic factors such as temperature, climate, soil pH, moisture, organic matter also including nutritive elements such as nitrogen and phosphorus (Cao et al., 2016; Wang et al., 2018; Chernov, Zhelezova, 2020). These biotic and abiotic factors are very dynamic and consist of many elements that can interact and influence microbial communities in the soil. Some studies have elucidated that the interaction between microbial communities and soil biotic and abiotic factors functions as an integrated impact of climate-soil-plant factors on the soil microbiome (Jiménez et al., 2019; Pingel et al., 2019). More so, soil microbial communities react primarily in response to changes in plant-soil interactions (Yao et al., 2018). Therefore, these microbial communities are essential in order to maintain homeostasis of the entire rhizospheric ecosystem (Raaijmakers et al., 2009).

Afforestation and soil microorganisms

Afforestation has a very important role in the functioning of rhizospheric ecosystems, it improves soil conditions and promotes soil development, especially in degraded ecosystems with an extremely poor environment (Ren et al., 2017; El Amrani, Amraoui, 2018). In addition, soil microbes react quickly to afforestation, which causes a huge increase in microbial proliferation (van der Wal et al., 2006). Dominant bacterial phyla, both Proteobacteria, Bacteroidetes, have been shown to be significantly more abundant in woodland than in abandoned land (Baldrian, 2017; Ren et al., 2018). In addition, the development of fungal communities also shows a significant increase after afforestation (Wallander et al., 2013; Gunina et al., 2017) in response to the improvement of the chemical properties of the soil as in the case of the conversion of abandoned land into forest (Yang et al., 2018).

However, natural forest ecosystems maintain greater soil microbial diversity than monoculture afforestation (Monkai et al., 2018). Some studies have shown that Ascomycota responded positively to land use change while Basidiomycota responded negatively (Ren et al., 2018). Also, ecosystems that contain a mixture of different plant genera have the potential to exhibit greater microbial community heterogeneity than single species plantations (Carnovale et al., 2019). From this proposition, it can be concluded that changes due to afforestation type may be related mainly to fungal phyla. Finally, this shows that maintaining the variability of plant species during afforestation greatly contributes to the conservation of the microbial diversity of the soil.

Plant species and soil microorganisms

The effect of afforestation on microbial communities may be due to the nature and diversity of plant species. In addition, plant species have been shown to significantly influence the composition and microbial structure of the soil (Yang et al., 2018). Therefore, the structure and function of the soil microbial community are often shown to be spatially associated with the composition, richness and biomass of plant communities (Gömöryová et al., 2013; Carnovale et al., 2019), as well as with stages of plant growth (Sheng et al., 2017). In addition, it has been believed that the soil microbiota responds quickly to variations in plant species (Yang et al., 2018) due to direct interaction between plant roots and soil microorganisms (Gömöryová et al., 2013). But this effect is not homogeneous and it is more pronounced on fungal communities than on bacterial communities (Carnovale et al., 2019). However, in addition to the direct effect of plant species on soil microbial communities, the structure and function of plant communities can indirectly influence (inhibit or stimulate) these microbial communities by altering the physical and chemical properties of the soil (Shen et al., 2013; El Amrani, 2017; Yang et al., 2018). Therefore, plant roots exert a strong impact on soil pathogens and beneficial microorganisms in the rhizosphere by producing exudates as well as secondary metabolites (Feng et al., 2019). Therefore, the enrichment of plant biodiversity plays a vital role in maintaining the microbial composition of the soil, which is not the case with monocultures. This conclusion is confirmed by the works of Schmid et al. (2019) who has tested, over the course of 11 years, soil bacterial communities developed under plant monocultures and mixtures. These works confirm that richness in plant species positively affects the composition and diversity of microbial communities.

Rhizospheric bacterial communities are considered cosmopolitan and colonize all biogeographical regions (Hanson et al., 2012). However, their activities and their diversities as well as their distributions are controlled by several parameters of the environment; among these factors, the plant figures as the major factor that controls them (Kumar et al., 2017). Some research has found conflicting results regarding prediction of microbial diversity by plant diversity when examining their relationships at large spatial scales (Liu et al., 2020). However, microbial communities are composed of groups that differ in their behavior. In this regard, we cite the obligate pathogenic

or symbiotic microorganisms, the life cycles of which can only be completed in the presence of their specific host such as obligate endophytes (Sally, David, 2008; Nair, Padmavathy, 2014; Glick, 2020). Another example can be seen in the case of ectomycorrhizae, most of which present host-symbiont specificity (Kernaghan et al., 2003). According to these two examples, we can only admit that parental control exerted by plant diversity influences the activity and microbial diversity of the rhizosphere. However, the degree of this control differs by several parameters mainly including the nature of microbial groups, plant species, and also soil and climatic conditions (Bargali et al., 2018; D'Acunto et al., 2018; Malard, Pearce, 2018). This explains the sometimes modest correlations between microbial richness and plant diversity (Liu et al., 2020).

Bulgarelli et al. (2015) used the term 'domestication' of bacterial communities by plant roots to explain the dominance of three bacterial families Comamonadaceae, Flavobacteriaceae and Rhizobiaceae in the barley root microbiota. On the other hand, a broad conservation of the composition of the root bacterial microbiota has been found in *Arabidopsis thaliana* and related species extending over 35 Ma within the family Brassicaceae (Schlaeppi et al., 2014). These results mean that the host plant determines and maintains its bacterial procession. This control of the soil microbial diversity by the plants is carried out mainly by the process of rhizodeposition (root excretion of photosynthesis-derived organic compounds) (Jones et al., 2009). These exudates can influence this microbial community either through trophic selection (trophic substances used by specific microbial groups) (Mansouri et al., 2002), biochemical selection (substances that stimulate or inhibit the proliferation of a given microbial group) (Rosier et al., 2018) or by chemotaxis (substances that attract targeted microbial groups to the roots of the plant) (Scharf et al., 2016).

Litter and soil microorganisms

The main methods by which plant communities affect soil chemical properties and subsequently microbial communities are primarily root and leaf litter, and root exudates (Zverev et al., 2016). Trees produce the majority of the waste deposited on the ground, in addition to a very large part of root exudates and dead roots under the ground (Gömöryová et al., 2013), which provides different inputs in quantity and quality (Yao et al., 2018). It is essential to claim that trees influence the soil microbiota basically in the same way as other plants, but their effect is potentially stronger due to the greater input biomass (Gömöryová et al., 2013). Therefore, the difference in the quantity and quality of litter and exudate inputs, different species and plant communities, modulates and causes a change in soil microbial communities (Santonja et al., 2018) even at the regional scale (Chen et al., 2015a).

Likewise, several previous studies have reported that differences in litter quality between tree species affect the abundance and composition of bacterial and fungal communities in the soil (Santonja et al., 2018; Pingel et al., 2019). In addition, differences in the quality of the litter occur in the nature of the inputs; such as the leaching of dissolved organic matter and nutrients, and the exudation of different kinds of ions and organic compounds (Yang et al., 2018). These variations can alter the rate and speed of fundamental soil processes, such as nutrient cycling and carbon dynamics, differently (Carnovale et al., 2019). Consequently, the greatest effect of plant species on the chemical properties of the soil is observed in the topsoil corresponding to the greatest amount of organic matter introduced (Kooch et al., 2017). From these results, it is clear that the quality and quantity of litter entering the soil are a determining factor in the existence of microbial communities and needs to be further investigated.

Secondary metabolites and soil microorganisms

Secondary metabolites are another component of plant litter of particular interest to soil ecosystems and exert a major effect on their edaphic microflora, especially in forest soils where complex phenol content is significantly higher (Yang et al., 2018). Similarly, Santonja et al. (2018) showed a contrasting activity of bacterial and fungal communities in response to the diversity of plant litter in a Mediterranean forest. These authors and others have shown that secondary metabolites repress biomass and the activity of microbial communities (Chomel et al., 2016; Santonja et al., 2018). Likewise, Chomel et al. (2014) showed a strong inhibitory effect of phenolic compounds, depending on the concentration, on fungal biomass in a Mediterranean pine forest. On the other hand, Amaral and Knowles (1998) reported the presence of monoterpenes negatively affecting the growth and activity of certain soil microbial groups while having a positive effect on other groups. However, knowledge of the effects of secondary metabolites on the activity and richness of soil microbial communities is still very limited.

Soil pH and microorganism communities

The change in pH is also a consequence of the biogeochemical interaction and has a major effect on the composition and activity of the soil microbial community. Therefore, the pH represents the primary metabolic control of microbial communities (Zhalnina et al., 2015). This control can be direct, by modulating the thermodynamics and kinetics of redox reactions and microbial respiration thereafter; or indirect by determining salinity and nutrient bioavailability through determination of proton chemical activity, mineral dissolution and precipitation, and other geochemical reactions (Bethke et al., 2011). On the other hand, soil pH describes the extracellular enzymatic activities and the rate of decomposition of organic matter (Jin Q., Kirk, 2018).

It has been reported that changes in the composition and diversity of microbial communities are positively correlated with variation in soil pH and that this variation controls their spatial distribution in the rhizosphere (Shen et al., 2013). This distribution was lower in monoculture plantations than in natural forests (Monkai et al., 2018). As reported in the study of Chen et al. (2015b), soil acidification decreased soil microbial respiration in forest ecosystems. These results suggest that reducing soil pH can lead to decreased biodiversity, rates of biogeochemical cycling, and ecosystem functioning (Chen et al., 2015b). Unlike bacterial communities, soil acidification has a slightly favorable effect on the richness of fungi in forest ecosystems (Rousk et al., 2011). Thus, the advanced knowledge of these interactions (pH-fungi-bacteria) can be a very powerful tool to mitigate negative effects caused by pathogenic fungi or bacteria by increasing or decreasing soil acidity.

Climate and soil microorganisms

The climatic conditions of soil ecosystems constitute one of the most determining parameters of the distribution of microbial communities. Previous research has confirmed that the spatial variation of soil microbial biomass depends on the spatial heterogeneity of climatic conditions (Xu et al., 2018). This justifies the use of microbiological properties as better indicators of soil quality, in particular the great capacity of microbial communities to react quickly to environmental changes (Marinari et al., 2006). As an example, several studies have reported that the mean annual temperature and mean annual precipitation show a positive correlation with microbial abundance and diversity (Cao et al., 2016; Tu et al., 2016). Also, low soil moisture and dry conditions during the summer drought period have a negative effect on microbial diversity and richness. These types of conditions can make a specific selection through the selection of drought resistant taxa such as fungi with lower nutritional requirements and higher water acquisition capacity or Gram positive bacteria (Manzoni et al., 2012; Xi et al., 2018).

From these results and the fact that soils belonging to the same climatic types have similar properties, we can conclude that climatic factors are of great importance for biodiversity and the richness of microbial biomass in the soil. It also suggests that soil microbes could be used as a more precise indicator of soil ecosystem characteristics.

Soil depth and soil microorganisms

Little is known about the effects of the physical properties of soil on the plant-microorganism interaction. However, the physical properties of soil have been reported to cause profound changes in soil microbial communities (Thoms et al., 2010; Xu et al., 2018). In addition to the physical properties of soil, the biomass and activities of fungal and bacterial communities also change at different depths of the soil profile (Carnovale et al., 2019). This vertical distribution reveals that fungi predominate in the topsoil of the soil, generally between 0 and 10 cm deep, and bacteria and actinomycetes predominate deep soils between 40 and 100 cm deep (Yao et al., 2018).

Nevertheless, it remains necessary to understand how physical properties, especially mechanical ones, can influence the microbiome residing in the soil and what mechanisms the microbiome can use to combat these types of stresses.

Conclusion

Microbial biodiversity is essential for improving sustainable plant growth and maintaining homeostasis of the entire rhizospheric ecosystem. In return, maintaining and enriching plant biodiversity greatly contributes to the conservation of soil microbial diversity. However, this balance depends and/or at the same time affects the biogeochemical cycle of the soil. Taken together, these interactions explain the complexity of understanding the dynamics of the rhizospheric microbiome. Hence the importance of such a study that could inform future work aimed at researching the interactions between microbial communities and other soil components in order to improve the management of resources and the productivity of rhizospheric ecosystems.

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Selective cultivation of bacterial strains with lipolytic and hydrocarbon-oxidizing activity from bottom sediments of the Ob River, Western Siberia

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Abstract. Bacteria play a key role in biogeochemical cycles in natural and anthropogenic ecosystems. In river ecosystems, bacteria intensively colonize silt sediments. Microorganisms are essential for energy conversion, biogeochemical nutrient cycling, pollutant degradation, and biotransformation of organic matter; therefore, bottom sediments can be a source of metabolically diverse microorganisms, including those with promise for industrial biotechnologies. The aim of this work was to isolate and study pure cultures of microorganisms – producers of industrially important enzymes and decomposers of organic matter – from bottom sediments of the Ob River. Pork fat and diesel fuel were used as substrates to obtain enrichment and pure cultures for selective cultivation of bacteria with lipolytic and hydrocarbon-oxidizing activity. A total of 21 pure cultures were isolated. The phylogenetic position of the obtained bacterial isolates was determined based on the analysis of 16S rRNA gene sequences. The strains isolated on selective media belonged to representatives of the genera *Pseudomonas* and *Aeromonas* (*Gammaproteobacteria*), and the genus *Microvirgula* (*Betaproteobacteria*). The ability of strains to grow on culture media containing pork fat, olive oil and diesel fuel was analyzed. The lipolytic activity of the isolates was evidenced by cultivation on a diagnostic medium containing 1 % tributyrin. The phylogenetic and metabolic diversity of the cultivated non-pathogenic bacterial strains with lipolytic and oil-oxidizing activity revealed in the study indicates the biotechnological potential of the isolates. The most promising strains were *M. aerodenitrificans* sp. LM1 and *P. lini* sp. KGS5K3, which not only exhibited lipolytic activity on the diagnostic medium with tributyrin in a wide temperature range, but also utilized diesel fuel, pork fat and olive oil.
Key words: microorganisms-decomposers; phylogenetic diversity; producers; lipolytic activity; organic substrates; biotechnological potential.

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Селективное культивирование бактериальных штаммов с липолитической и нефтеокисляющей активностью из донных осадков реки Оби в Западной Сибири

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Аннотация. Бактерии играют ключевую роль в биогеохимических циклах природных и антропогенных экосистем. В речных экосистемах бактерии, как правило, интенсивно заселяют илистые отложения. Микроорганизмы имеют важное значение в преобразовании энергии и биотрансформации органических веществ. В связи с этим донные отложения, богатые органикой, могут являться источником выделения метаболически разнообразных микроорганизмов, в том числе перспективных для промышленных биотехнологий. Целью данного исследования было выделение и изучение чистых культур микроорганизмов – продуцентов промышленно значимых ферментов и деструкторов органических веществ из донных осадков р. Оби. В качестве субстратов для выделения накопительных и чистых культур использовали свиной жир и дизельное топливо для селективного культивирования бактерий с липолитической и углеводородокисляющей активностью. Всего получена 21 чистая культура. Филогенетическое положение бактериальных изолятов определено на основе анализа последовательностей генов 16S рРНК. Выделенные на селективных средах штаммы оказались представителями родов *Pseudomonas* и *Aeromonas* класса *Gammaproteobacteria* и рода *Microvirgula* класса *Betaproteobacteria*. Изучена способность штаммов к росту на плотных питательных средах со свиным жиром, оливковым маслом и дизель-

ным топливом. Липолитическая активность штаммов подтверждена культивированием на диагностической среде с трибутирином. Обнаруженное в ходе исследований филогенетическое и метаболическое разнообразие культивируемых непатогенных бактериальных штаммов с липолитической и нефтеокисляющей активностью указывает на биотехнологический потенциал выделенных нами изолятов. Наиболее перспективными оказались штаммы *M. aerodenitrificans* sp. LM1 и *P. lini* sp. KGS5K3, которые не только проявили липолитическую активность на диагностической среде с трибутирином в широком диапазоне температур, но и утилизировали такие сложные органические субстраты, как дизельное топливо, свиной жир и оливковое масло.
Ключевые слова: микроорганизмы-деструкторы; филогенетическое разнообразие; продуценты; липолитическая активность; органические субстраты; биотехнологический потенциал.

Introduction

Bacteria play a significant role in the biogeochemical cycles in natural and anthropogenic ecosystems. In river ecosystems, bacteria intensively colonize silt sediments (Araya et al., 2003). The river microbial network is a directional linear branched structure shaped by the river flow. Microorganisms are transferred from the water column to the underlying sediments and enrich them (Brown et al., 2011; De Oliveira, Margis, 2015; Mansour et al., 2018; Wang L. et al., 2018). The branched structure of the river ecosystem contributes to accumulation of bacteria from surrounding lands, including urban and industrial areas, wastewater treatment plants and agricultural lands, which also contain soluble components (Mansour et al., 2018). These include organic matter, nutrients or toxic compounds, and metals, which affect the activity and abundance of heterotrophic bacteria in bottom sediments (Fischer et al., 2002).

Microorganisms are crucial for energy conversion, biogeochemical nutrient cycling, pollutant degradation, and biotransformation of organic matter; therefore, bacteria can be used as bioindicators of aquatic ecosystems (Wei et al., 2008; Chen et al., 2018). For example, the abundance of *Nitrospirae*, *Betaproteobacteriales*, *Chloroflexi*, and *Sphingobacteriales* represantatives was found to increase in proportion to an increase in the concentration of nitrogen, which shows high concentrations due to anthropogenic load. An increased proportion of *Nitrospirae*, *Sphingobacteriales* (*Bacteroidetes*) and *Spirochaetes* and a generally decreased abundance of *Actinobacteria* were observed in sediment communities of river ecosystems located near wastewater treatment plants, which indicates the impact of wastewater (Sagova-Mareckova et al., 2021).

Thus, characterization of the composition of bacterial communities in the water column and river sediments, as well as the response of microbial communities to environmental changes, can yield valuable information to explore microbial interrelations and assess the environmental risk (Psenner et al., 2008; Wang J. et al., 2016). In addition, bottom sediments can be a source of metabolically diverse microorganisms, including those promising for industrial biotechnologies.

Works that address the species composition and functions of microbial communities in river ecosystems are few in number as compared, for example, with those related to ecosystems of salt lakes or seas. Microbiological studies of rivers flowing through the territory of Russia cover mainly their sanitary and epidemiological status (Shornikova, 2008). A.I. Kopylov and D.B. Kosolapov investigated distribution of bacterioplankton in the lower reaches of the Ob River and provided measurements of the specific growth rate, and the abundance and distribution of biomass in different parts of the river (Kopylov, Kosolapov, 2011). Other works related to microbiological monitoring of the Ob River studied the abundance and distribution of some metabolic groups of microorganisms (Savichev et al., 2015), including those resistant to antibiotics and phenol (Shornikova, Arslanova, 2020). Yet the species diversity and physiological characteristics of the native microflora have been poorly studied.

The Ob River flows through the territory of Western Siberia and ranks among the first in terms of length, water content and catchment area among Eurasian rivers. In the Siberian region, the Ob River is exposed to the greatest anthropogenic load, including demographic, agricultural and industrial impact; its water quality indicators for the content of certain metals and oil products are considered critical (Koronkevich et al., 2019). Therefore, the study of microbial communities in the water column and bottom sediments is of relevance, including the search for biotechnologically promising microorganisms – decomposers of organic matter.

The aim of this study was to isolate pure cultures of microorganisms-decomposers from bottom sediments of the Ob River, analyze their ability to utilize various organic substrates, and detect their lipolytic and hydrocarbon-oxidizing activity in different cultivation conditions.

Material and methods

Bottom sediment samples were collected in July 2020 in the middle reaches of the Ob River near the following settlements: Molchanovo (57.601429° N, 83.7824851° E), Kolpashevo (58.30456° N, 82.90774° E), Kargasok (59.06722° N, 80.84963° E). Sediments (sandy deposits) sampled from a depth of 1.5 m were put into sterile plastic test tubes and stored at +4 °C. The pH level of the water at the sampling sites was shifted to slightly alkaline pH values (from 7.5 to 8.6) (Frank et al., 2021).

Strains – decomposers of organic matter and producers of biotechnologically significant enzymes – were isolated by selective cultivation on culture media for lipolytic and hydrocarbon-oxidizing microorganisms. Initial enrichment cultures from each sampling site were obtained on a selective mineral medium containing pork fat (1 % of the medium volume) used as the only carbon source (Gerasimchuk et al., 2020) and on the medium used for hydrocarbon-oxidizing

bacteria supplemented with 1 % diesel fuel, as described in (Frank et al., 2020). Samples taken in an amount of 0.5 ml from each site were inoculated in 50 ml of the liquid medium (pH 7.5) containing pork fat in 120 ml glass vials and cultivated at +28 °C in oxygen. The first inoculation to obtain hydrocarbon-oxidizing microorganisms was performed by limiting dilutions in 7 ml of the liquid medium in 15 ml glass penicillin vials. After that, the resulting enrichment cultures were inoculated on agar media of similar composition to obtain individual colonies. The colonies grown on plates with enrichment cultures were transferred to GRM broth (8 g/l pancreatic hydrolyzate of fish meal, 8 g/l enzymatic peptone, 4 g/l sodium chloride, pH 7.0–7.4). Then, the obtained aerobic strains were cultivated on Petri dishes with GRM broth at +28 °C.

The morphology of enrichment and pure cultures was analyzed by phase contrast microscopy (Biomed 6, Russia) using ×100 immersion lens.

The ability of strains to oxidize petroleum products was estimated using a liquid culture medium (g/l: KH_2PO_4 – 1.5, K_2HPO_4 – 0.75, NH_4Cl – 1.0, NaCl – 2.5, $MgSO_4 \cdot 7H_2O$ – 0.2, yeast extract – 0.5) supplemented with 1 % diesel fuel as an organic substrate. The inoculations were performed in 15 ml penicillin vials filled with the medium to 1/3 (5 ml). Incubation proceeded at +28 °C. The growth was assessed by medium turbidity and using microscopy. To confirm the hydrocarbon-oxidizing activity of individual strains, inoculations of the dense mineral medium without yeast extract (Gerasimchuk et al., 2020) were performed on Petri dishes with the addition of 0.1 ml of diesel fuel spread together with the inoculum on the medium surface.

The ability to utilize animal and vegetable fat was studied using a mineral culture medium (Gerasimchuk et al., 2020) containing 1 % pork fat or 1 % olive oil. The cultivation procedure was as follows: 0.25 ml of molten sterile pork fat or olive oil was spread on Petri dishes filled with 25 ml of the agar mineral medium; the inoculum was placed in a droplet of pork fat and spread on the surface of the medium using a spatula or a bacterial loop.

Lipolytic activity was detected using a diagnostic medium (tributyrin agar) containing 0.5 % (w/v) peptone, 0.3 % (w/v) yeast extract and 1.5 % bacteriological agar (pH 7.0) supplemented with 1 % tributyrin (Ramnath et al., 2017). Tributyrin is an ester composed of butyric acid and glycerol. Tributyrin agar is mainly used to detect lipolytic activity in bacteria (Mourey, Kilbertus, 1976). Cultures were incubated at +28, +25, and +4 °C. After 24 or 48 h of incubation, hydrolysis zones (transparent halos) could be observed around the colonies.

The phylogenetic position of the obtained strains was determined by sequencing and analyzing 16S rRNA gene sequences. Genomic DNA was isolated from cultures using the Biolabmix kit (DU-50) in accordance with the manufacturer's recommendations (http://biolabmix.ru/). For amplification of bacterial 16S rRNA genes, which are universal phylogenetic markers, primers 27F (DeLong, 1992) and 1492R (Weisburg et al., 1991) were used. A 50 µl PCR mixture contained 1x PCR buffer (Biolabmix), 2.5 mM $MgCl_2$ (Biolabmix), 0.2 mM dNTP mixture (Biolabmix), 10 pM of each primer (Sintol), 0.7 U thermostable HS-Taq polymerase (Biolabmix), 3 µl of template DNA (at a concentration exceeding 50 ng); the mixture was brought to the final volume with sterile deionized water.

The 16S rRNA genes were amplified in accordance with the procedure described in (Gerasimchuk et al., 2010). Sequencing of the obtained DNA sequences was performed using a genetic analyzer NANOFOR-05 in Scientific and Production Company "Sintol", Moscow. To obtain a nearly complete 16S rRNA gene sequence, the forward primer 27F (DeLong, 1992), the reverse primer 1492R (Weisburg et al., 1991), and the BacV3F primer (Muyzer et al., 1993) were used.

The obtained nucleotide sequences of the 16S rRNA genes were analyzed using the BIOEDIT sequence editor (http://www.jwbrown.mbio.ncsu.edu), the BLAST program in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov), and the SILVA database classifier (http://www.arb-silva/aligner/de). Chimeric sequences were detected using the DECIPHER package (http://www2.decipher.codes/FindChimeras.html). The obtained nucleotide sequences of the 16S rRNA gene fragments were deposited in the GenBank database under the numbers: OM212652, OM212653, OM212656–OM212659, OM212664–OM212671.

Results and discussion

Enrichment and isolation of pure cultures

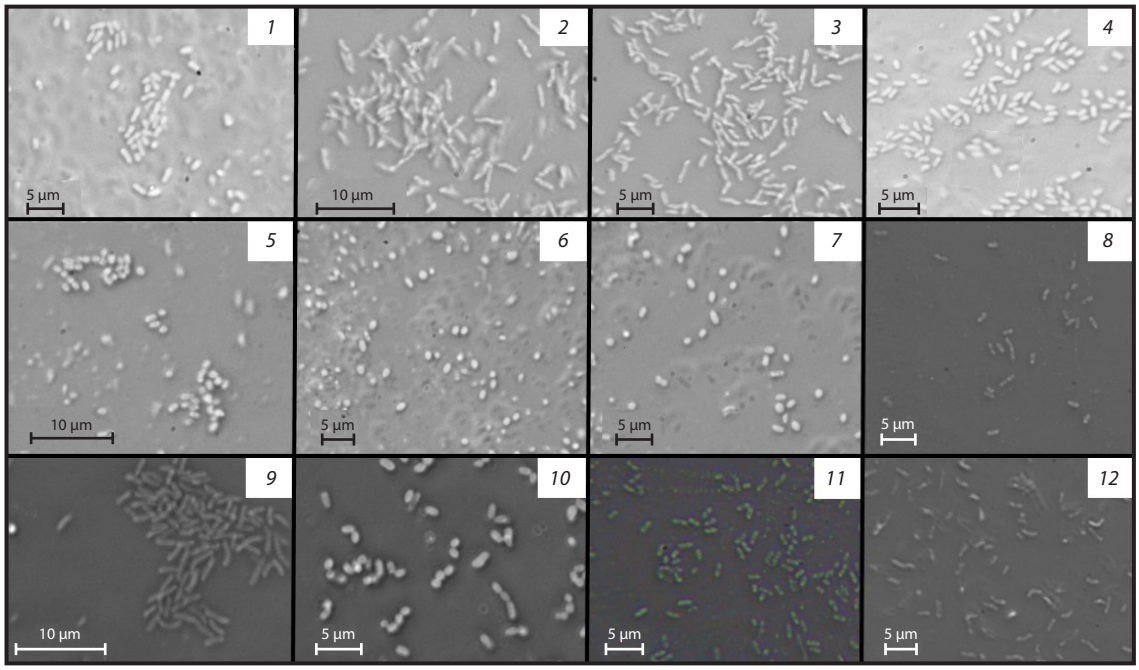
For isolation of bacterial strains – producers and decomposers of organic matter – samples of bottom sediments from the middle reaches of the Ob River and selective culture media for lipophilic and hydrocarbon-oxidizing microorganisms were used. Morphologically homogeneous pure cultures were isolated from enrichment cultures, which showed high abundance and morphological diversity of cell forms (see the Figure). Strains that exhibited stable growth on GRM agar were used for further studies. In total, 9 pure cultures were obtained from separate colonies isolated on the medium used for hydrocarbon-oxidizing bacteria, and 12 pure cultures were isolated on the medium used for lipolytics.

Phylogenetic analysis

Analysis of the 16S rRNA gene fragments showed that the strains belong to Proteobacteria (*Gammaproteobacteria* and *Betaproteobacteria*) (see the Table). Proteobacteria often dominate in the bacterial communities in water and bottom sediments, and their proportion in sediments is typically higher than that in water (Dai et al., 2016; Zhang et al., 2019).

Most of the analyzed strains were representatives of *Pseudomonas* and *Aeromonas* (*Gammaproteobacteria*). All the obtained and analyzed fragments of the 677–1445 bp DNA sequences showed a high percentage of similarity (99.48–100 %) with sequences of typical strains of microorganisms deposited in the GenBank NCBI database.

A part of the strains was related to opportunistic pathogens belonging to hazard group II according to the WHO classification (https://bacdive.dsmz.de/). The detected pathogens included all *Aeromonas* strains related to *A. veronii* (LKar2 and LKar3), *A. hydrophila* (LM7 and KLP3), as well as *E. coli* (LKol1) and *P. putida* (LM3 and LM4). Most of the pathogenic strains were isolated from lipophilic enrichment cultures.



Micrographs of cells in the pure cultures: *1*, LM7 strain; *2*, LM6 strain; *3*, LM8 strain; *4*, KGS3Ps1 strain; *5*, LKol1 strain; *6*, LM3 strain; *7*, LM4 strain; *8*, KGS5k2 strain; *9*, KGS3Ps2 strain; *10*, KGS5k3 strain; *11*, KGS5k1 strain; *12*, LKol3 strain.

Phase contrast microscopy, ×1000 magnification.

Conditionally pathogenic microorganisms (enterobacteria related to *Serratia marcescens*, *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, *K. huaxiensis*, *Enterobacter cloacae*, *Raoultella ornithinolytica*, *Morganella morganii*) grown on the mineral medium containing pork fat were isolated in our previous studies when isolating pure cultures of lipophilic microorganisms from wastewater treatment plant effluent and food industry wastewater (Gerasimchuk et al., 2020). Bacterial lipases involved in such metabolic processes as hydrolysis and lipid modification can be virulence factors for some phylogenetic groups, which explains numerous pathogens found among lipophilic bacteria (Bender, Flieger, 2010; Kovacic et al., 2019).

Other bacteria were related to microorganisms that are non-human pathogens. Most of the strains were representatives of the genus *Pseudomonas*. The genus *Pseudomonas* includes a large group of Gram-negative bacteria that exhibit a great metabolic diversity, which allows them to utilize a wide range of organic compounds and play important ecological roles in the carbon cycling. Pseudomonas are ubiquitous in a wide variety of ecosystems and include many pathogenic human, animal, and plant species (Peix et al., 2009), as well as mutualistic species, which include the most remarkable representatives of biocontrol strains that protect plants from pathogens (Ramette et al., 2011; De Vrieze et al., 2015). Pseudomonades can degrade various lipids and lipid-containing compounds (Pabai et al., 1996; Lee, Rhee, 2008; Yang J. et al., 2009; Fendri et al., 2010), as well as oil hydrocarbons (Barathi, Vasudevan, 2001). Representatives of the genus *Pseudomonas* are often found in river ecosystems, which is evidenced by molecular studies (Cyriaque et al., 2020) and cultural methods (Pellett et al., 1983; Pirnay et al., 2005), including isolation of new pseudomonades from oil-contaminated bottom sediments in China (Li et al., 2020), dioxin-contaminated bottom sediments in Texas (Iyer et al., 2017), bottom sediments in India (Sudan et al., 2018), etc.

Analysis of the 16S rRNA gene sequencing performed for the Mol4a strain showed 100 percentage of similarity with sequences of *P. veronii* strains from various habitats (activated sludge, hydrocarbon-contaminated groundwater, contaminated sediments, etc.), and 99.82 percentage of similarity with the *P. veronii* type strain isolated from mineral waters (Elomari et al., 1996). The KGS3Ps2 strain was found to be closely related to *P. protegens* (LS999205) isolated from soil, which together with representatives of *P. veronii* belongs to the *Pseudomonas fluorescens* group. *P. baetica* belongs to the same taxonomic group, and its 16S rRNA gene showed 100 percentage of sequence similarity with the KGS3Ps1 strain. The type strain of the above bacterium is a pathogen for sole (López et al., 2012).

The KGS5k1 strain showed the highest percentage of similarity (99.86 %) with an undescribed strain isolated from soil and referred to as *P. brassicacearum* (KT695825), and 99.5 percentage of similarity with a valid strain *P. chlororaphis* (CP027720) isolated from fluviatile loam and related to the *Pseudomonas chlororaphis* taxonomic group. Comparison of the nearly complete 16S rRNA gene sequence showed a similar percentage of similarity between the Mol4k12 strain and the type strains of different species, namely, *P. fildesensis* (MK859934) and *P. extremaustralis* (KX186942), which are closely related to representatives of *P. fluorescens*. KGS5k2, KGS5k3, and KGS5k8 strains showed 100 % homology with the 16S rRNA gene of *P. lini* (NR_029042) isolated from rhizosphere soil (Delorme et al., 2002).

The genus *Pseudomonas* is one of the taxonomically most complex genera (Parte, 2014). Although the 16S rRNA gene is

Phylogenetic position of bacteria isolated from bottom sediments

Strain	GenBank access. number	Sequence length	Percentage of similarity	Closely related valid organism (GenBank)	Source	Phylogenetic affiliation
Pure cultures isolated from lipophilic enrichment cultures on mineral medium containing pork fat						
LM1	OM212667	1364	100	*Microvirgula aerodenitrificans* (MT367755)	Animal intestines	Beta, Nies
LM2	–	613	100			
LM3	–	681	100	*Pseudomonas putida* (KX083533)	No data	Gam, Pseu
LM4	–	679	100			
LM6	–	617	100	*M. aerodenitrificans* (MT367755)	Animal intestines	Beta, Neis
LM7	OM212659	680	100	*Aeromonas hydrophila* (MG428802)	Fish intestinal mucosa	Gam, Aero
LM8	OM212666	1407	100	*M. aerodenitrificans* (MT367755)	Animal intestines	Beta, Neis
LKar2	OM212656	686	100	*A. veronii* (NR_118947)	Human sputum	Gam, Aero
LKar3	OM212658	679	100			
LKol1	–	470	100	*Escherichia coli* (AP022215)	Wastewater treatment plant effluent	Gam, Enter
LKol2	OM212669	1364	100	*M. aerodenitrificans* (MT367755)	Animal intestines	Beta, Neis
LKol3	–	621	100			
Pure cultures isolated from enrichment cultures on hydrocarbon-containing medium						
KGS5k1	OM212664	1413	99.86	*P. brassicacearum* (KT695825)	Soil in the USA	Gam, Pseu
KGS5k2	–	631	100	*P. lini* (NR_029042)	Rhizosphere soil	
KGS5k3	OM212670	1345	100			
KGS5k8	OM212671	1379	100			
KGS3Ps1	OM212652	1445	100	*P. baetica* (KU921565)	Sediments from a lake in India	
KGS3Ps2	OM212653	1413	99.83	*P. protegens* (LS999205)	Soil	
KLP3	OM212657	677	100	*A. hydrophila* (MT572504)	Contaminated soil	
Mol4A	OM212665	1108	100	*P. veronii* (MH669341)	Pine tree	
Mol4K12	OM212668	1260	99.92	*P. fildesensis* (MK859934)/ *P. extremaustralis* (KX186942)	Antarctic soil/ non-perennial reservoir	

Note. Beta, Nies – *Betaproteobacteria*, *Neisseriales*; Gam, Pseu – *Gammaproteobacteria*, *Pseudomonadales*; Gam, Enter – *Gammaproteobacteria*, *Enterobacterales*; Gam, Aero – *Gammaproteobacteria*, *Aeromonadales*.

a universal phylogenetic marker in the bacterial classification system, the analysis of this gene alone does not allow differentiation of closely related bacterial species. Recent studies have shown that multilocus sequence analysis (MLSA) performed for four housekeeping genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*) enables species identification and facilitates strain identification in *Pseudomonas* (Mulet et al., 2012). Thus, a more precise determination of the phylogenetic position of the isolated *Pseudomonas* strains requires analysis of additional molecular markers (for example, *gyrB* and *rpoD* genes).

Analysis of 16S rRNA gene sequences showed that LM1, LM2, LM6, LM8, LKol2, and LKol3 strains are identical and can be assigned to the genus *Microvirgula*, the class *Betaproteobacteria*. Interestingly, we earlier isolated the identical 768 bp sequence (GenBank accession number MT476921) that belong to bacteria of the genus *Microvirgula* from food industry waste (Gerasimchuk et

al., 2020). Representatives of the genus *Microvirgula* grow well in aerobic and anaerobic conditions, have an atypical respiratory type of metabolism, and use oxygen and nitrogen oxides as the final electron acceptors (Patureau et al., 1998). The genus *Microvirgula* was first described by Patureau et al. (1998) and was characterized as a new denitrifying bacterium *M. aerodenitrificans* isolated from activated sludge. At present, the genus *Microvirgula* includes two species. The other representative of *M. curvata* was isolated from hydrocarbon-contaminated soil (Subhash et al., 2016) and included one strain. Comparison of the sequenced fragments of the 16S rRNA genes of LM1, LM2, LM6, LM8, LKol2, and LKol3 strains showed that their sequences are identical and exhibit 100 % homology with the strain of *M. aerodenitrificans* (MT367755) isolated from the intestines of wild animals. They also showed 99.79 and 99.86 percentage of similarity with *M. aerodenitrificans* Sgly2 isolated from activated sludge (Patureau et al., 1998) and the type strain, *M. aerodenitrificans* NBRC 15328 (AB680837) isolated from fresh water (Cleenwerck et al., 2003), respectively.

Lipolytic activity of numerous representatives of *Pseudomonas* was thoroughly investigated on various substrates, lipolytic enzyme genes were studied and cloned (Reetz, Jaeger, 1998; Bofill et al., 2010; Yang W. et al., 2015; Cai et al., 2016), and their hydrocarbon-oxidizing activity was confirmed (Muriel-Millán et al., 2019). At the same time, only lipolytic activity on diagnostic media was shown for representatives of the genus *Microvirgula*, and their lipolytic properties were not studied in detail. Yet the analysis of *Microvirgula* genomes available in the NCBI database revealed lipolytic enzyme genes. At present, data have been published on the genomes of two strains of *M. aerodenitrificans* (JHVK01000000 and CP028519) isolated from different bioreactors and one strain of *Microvirgula* (NZ_QLTJ01000000) with an unidentified phylogenetic position, which is a bacterial endophyte of rice. The search for lipolytic enzyme genes in the listed genomes revealed the presence of lipases and esterases. Additionally, data on sequences of *Microvirgul*a strains isolated from petroleum-contaminated habitats (the GenBank database, accession numbers KM357844, LT631813) indirectly indicate their hydrocarbon-oxidizing activity.

Detection of lipolytic activity of strains using a diagnostic medium

Non-pathogenic strains of different phylogenetic affiliation with nearly complete 16S rRNA sequences (see the Table) and different growth or morphology characteristics were used to study lipolytic activity. All the strains grew and formed hydrolysis zones on tributyrin agar after 24–48 h of cultivation at +28 °C, except for *P. veronii* sp. Mol4, which did not form hydrolysis zones, most likely due to the absence of lipolytic activity and the use of peptone, the component of the culture medium, as a growth substrate. In contrast to other strains with hydrolysis zones of about 3 mm, *P. protegens* sp. KGS3Ps2, *P. brassicacearum* sp. KGS5k1, and *M. aerodenitrificans* sp. LM1 showed a more pronounced lipolytic activity in the form of complete hydrolysis reaction. Additionally, *P. protegens* sp. KGS3Ps2 and *P. brassicacearum* sp. KGS5k1 exhibited growth and lipolytic activity at +4 °C.

Study of the ability of strains to utilize organic substrates

In contrast to *Microvirgula* strains, *Pseudomonas* strains grown on GRM agar exhibited psychrotolerant properties and stable growth at +4 °C. None of the studied thermotolerant strains showed stable growth at +50 °C. There were no significant difference in biomass gain on GRM and tributyrin agar at +25 and +28 °C.

On dense media containing 1 % pork fat and 1 % olive oil, strains of *M. aerodenitrificans* sp. LM1 and *P. lini* sp. KGS5k3 were observed to grow at +25 and +28 °C. The inoculations on media containing pork fat and olive oil at +4 °C showed that animal fat and vegetable oil do not degrade or this process is constrained at low temperatures. *P. protegens* sp. KGS3Ps2 and *P. brassicacearum* sp. KGS5k1 did not grow on these media even at +28 °C despite their more pronounced lipolytic activity on diagnostic media.

Screening of strains on the selective medium containing 1 % diesel fuel showed that 5 out of 10 strains, namely, *P. protegens* sp. KGS3Ps2, *M. aerodenitrificans* sp. LM1, *P. fildesensis/extremaustralis* sp. Mol4K12, and *P. lini* spp. KGS5k3 and KGS5k8, are able to grow on a hydrocarbon-containing medium. To confirm the hydrocarbon-oxidizing activity of *M. aerodenitrificans* sp. LM1 and *P. lini* sp. KGS5k3 – the most promising decomposers – additional inoculations were carried out on a dense mineral medium containing diesel fuel as the only carbon source. The growth of strains was observed after less than 2 days. It should be noted that *P. lini* sp. KGS5k3 yielded more biomass.

Conclusion

The phylogenetic and metabolic diversity of cultivated non-pathogenic bacterial strains with lipolytic and hydrocarbon-oxidizing activity revealed in the study indicates biotechnological potential of the isolates. The most promising strains are *M. aerodenitrificans* sp. LM1 and *P. lini* sp. KGS5k3, which exhibited lipolytic activity on a diagnostic medium in a wide temperature range and utilized such complex organic substrates as diesel fuel, pork fat and olive oil. For the first time, the ability to oxidize petroleum products and grow on specific fat-containing substrates was shown for representatives of *M. aerodenitrificans*. Earlier, only lipolytic activity on diagnostic media was reported for *M. aerodenitrificans* (Patureau et al., 1998). The biotechnological potential of *M. aerodenitrificans* described in the literature indicates its ability for aerobic and anaerobic denitrification in waste treatment technologies using bioreactors (Patureau et al., 2001; Bouchez et al., 2009; Anderson et al., 2020). However, isolation of 16S rRNA phylotypes and pure cultures related to the genus *Microvirgula* from hydrocarbon-contaminated samples (Subhash et al., 2016; Sarkar et al., 2017) and waste effluents (Cea et al., 2015; Gerasimchuk et al., 2020), and our results on the growth obtained using media containing fat and oil products, indicate a wider biotechnological potential of these microorganisms.

No literature data were found on lipolytic activity of *P. lini*. Thus, we have shown for the first time lipolytic activity of representatives of this species using a diagnostic medium, and their ability to utilize oil products and animal fat.

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Effect of biogenic polyamines on sliding motility of mycobacteria in the presence of antibiotics

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Abstract. Nowadays, sliding is the least investigated mode of bacterial motility. Sliding is a process of passive movement on the surface of semi-liquid mediums which was originally described for mycobacteria and other bacterial species deprived of the organelles specialized for movement. Some mycobacteria are able to colonize surfaces, including tissues of macro-organisms, using glycopeptidolipids localized in the cell envelope for this aim. This is a serious problem for effective therapy of mycobacteriosis caused by nontuberculosis mycobacteria. Furthermore, animal tissues contain biogenic polyamines, which can increase tolerance of microorganisms to stresses, including antibiotics, and modulate cell motility. Therefore, studying mutual effects of biogenic polyamines and antibiotics on the expansion of mycobacteria is important for medicine. Mycobacterial strains, including the parent *Mycolicibacterium smegmatis* mc² 155 and strains containing single (Δ*rel*$_{Msm}$) or double (Δ*rel*$_{Msm}$Δ*relZ*) deletions, were used as the objects of this study. The content of glycopeptidolipids was determined using thin layer chromatography. Sliding motility was assessed by measuring the area of the sliding colony. The effectiveness of antibiotics was measured by comparison of the areas of sliding colonies in the presence of comparable concentrations of antibiotics. The polyamines spermidine and spermine had different effects on the sliding of mycobacteria through an increase or decrease in the colony areas. At the same time, polyamines had neither bactericidal nor bacteriostatic effects. The polyamines contained in the medium decreased the bactericidal effects of the antibiotics streptomycin or isoniazid, but enhanced the effects of DMNP, a synthetic analogue of the natural antibiotic erogorgiaene. Rifampicin was the most effective of all antibiotics investigated here. Moreover, we found that glycopeptidolipids are, apparently, not the only regulators of mycobacterial sliding.
Key words: mycobacteria; sliding motility; antibiotic susceptibility; biogenic polyamines.

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Влияние биогенных полиаминов на скольжение микобактерий в присутствии антибиотиков

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Аннотация. Из всех известных способов передвижения бактерий скольжение является наименее изученным. Впервые описанное для микобактерий и некоторых других бактериальных видов, скольжение представляет собой пассивный способ перемещения по поверхности полужидких питательных сред у видов, лишенных органелл движения. Несмотря на отсутствие механизмов перемещения, некоторые микобактерии способны быстро колонизировать поверхности, в том числе ткани многоклеточных организмов, за счет присутствия в составе наружного слоя их клеточной стенки гликопептидолипидов, регулирующих силу трения о поверхность при перемещении. Это представляет серьезную проблему для эффективной терапии микобактериозов, вызываемых нетуберкулезными микобактериями. Кроме того, ткани многоклеточных животных содержат биогенные полиамины, которые способны повышать устойчивость микроорганизмов к различным стрессам, в том числе к антибиотикам, и модулировать коллективное движение. Поэтому исследование совместного действия биогенных полиаминов и антибиотиков на процессы распространения микобактерий представляет большой интерес для медицины. В качестве объектов исследования использованы штаммы микобактерий, включая родительский штамм *Mycolicibacterium smegmatis* mc² 155, а также его производные, содержащие одинарную (Δ*rel*$_{Msm}$) или двойную (Δ*rel*$_{Msm}$Δ*relZ*) хромосомные делеции. Содержание гликопептидолипидов определяли с помощью метода тонкослойной хроматографии. Интенсивность скольжения оценивали путем измерения площади скользящей

колонии. Эффективность действия антибиотиков характеризовали сравнением площадей скользящих колоний в присутствии сопоставимых концентраций антибиотиков, выраженных в значениях, кратных минимальной подавляющей концентрации. Показано, что полиамины спермидин и спермин оказывают разнонаправленные эффекты на скольжение микобактерий по поверхностям полужидких сред, соответственно повышая или снижая площади колоний. При этом использовали концентрации спермидина и спермина, которые сами по себе не оказывали бактерицидный или бактериостатический эффект. Однако их совместное применение с антибиотиками стрептомицином или изониазидом приводило к снижению антибактериального эффекта, но усиливало действие синтетического аналога природного антибиотика эрогоргиаена (DMNP). Наиболее эффективным в этих условиях был рифампицин. Более того, нами установлено, что гликопептидолипиды, по-видимому, являются не единственными регуляторами скольжения микобактерий.
Ключевые слова: микобактерии; скольжение; антибиотикочувствительность; полиамины.

Introduction

Sliding is a passive way of spreading bacteria on semisolid plates, which was described in 1972 (Henrichsen, 1972). At the basis of this type of motility is the action of the expansive force that occurs when dividing cells press against each other. Bacteria are pushing each other and spreading on the surface of the plate in a layer of cells through releasing surfactants in environment (Hölscher, Kovács, 2017) or accumulating glycopeptidolipids (GPLs) in cell walls (Recht et al., 2000) to decrease friction on a solid surface. Sliding motility may be realized without flagella and pili. This type of motility is available for species that were previously considered immobile. For example, in 1999, it was observed that mycobacteria can spread on semisolid pales (Martínez et al., 1999). Later, a link was established between GPLs content in mycobacterial cell walls and sliding motility. Accordingly, a model was proposed under which GPLs hydrophobic tails localized in the outer layer of the cell wall are facing the environment and so are responsible for the hydrophobic surface of cells. This hydrophobic surface doesn't interact with hydrophilic agar plate enabling sliding for bacteria. In contrast, interaction between two hydrophobic surfaces promotes attraction between cell wall and polyvinyl chloride plates and biofilm formation (Recht et al., 2000).

Therefore, GPLs are considered to be the main factors of sliding motility in mycobacteria. However, sliding motility may be also modulated by many environmental factors like extracellular signaling molecules or plate conditions. For example, extracellular ATP secreted by damaged epithelial cells is a signaling molecule that inhibits the pulling movement of *Pseudomonas aeruginosa* (Nolan et al., 2015), whereas the polyamines (PA) putrescine and spermidine synthesized by *Escherichia coli* are required to initiate the swarming (Kurihara et al., 2009).

The role of polyamines as signaling molecules is important because these polycations are present in the cells and tissues of most living organisms, as well as water and soil. Human blood, skin, and mucous membranes also contain PAs, mainly cadaverine, spermidine, and spermine, the intracellular concentration of which can reach 2–10 mM (Gugliucci, 2004). Bacteria can synthesize putrescine, cadaverine and spermidine. PA synthesizing genes were also found in the genome of mycobacteria (Zamakhayev et al., 2018), but we have previously shown that mycobacteria can't synthesize their own polyamines (Zamakhaev, 2020). Nevertheless, they are able to transport these polycations from the external environment. Polyamines have a positive charge and so they are able to bind to negatively charged molecules inside the cells, primarily nucleic acids, and modulate replication, transcription, translation, and other cell processes. Bacteria, getting on tissues similar to semi-solid agar in their moisture, for example, mucous membranes, are able to slide through a medium rich in biogenic polyamines, which are able to get into cells and modulate intracellular processes.

Investigation of mycobacteria sliding is important because non-tuberculous mycobacteria, the cell walls of which contain GPLs, are the cause of lung and skin infections (Tran et al., 2019). Moreover, non-tuberculous lung infections (NTLIs) have received less attention, which is likely responsible for underestimation of the incidence data on TB in the United States (Strollo et al., 2015). There are no GPLs in the cell wall of *Mycobacterium tuberculosis*. That is why it's currently considered to be non-sliding. However, its cell wall contains phosphatidylinositol mannosides, phenolic glycolipids, as well as lipomannan and lipoarabinomannan (Tran et al., 2019). These lipids are also capable of creating a hydrophobic environment similar to that considered by the *M. smegmatis* sliding model.

Previously, we have shown that a synthetic analogue of the natural diterpene erogorgiaene, DMNP, along with widely used clinical antibiotics, has antimycobacterial activity, and its targets are the large and small alarmone synthetases Rel_{Msm} and RelZ, which are responsible for the intracellular level of alarmone guanosine tetraphosphate (p)ppGpp (Tkachenko et al., 2021). This makes the new compound effective against the formation of quiescent cells and a promising substance for the development of new antimycobacterial drugs. Therefore, DMNP has also been a subject for studying its possible effects on *M. smegmatis* sliding colonies. We compared the new compound with widely used antibiotics rifampicin, streptomycin, and isoniazid, and investigated the effect of biogenic polyamines. The latter substances are represented in the natural environments and so can have a cell protective effect on mycobacteria against antibiotics (Sarkar et al., 1995).

Materials and methods

Strains and growth media. Strains *Mycobacterium smegmatis* mc^2 155 were objects of this study. The strain without deletion was used as a control and is indicated on the graphs as 'WT'. Mutant strains with a single deletion of the rel_{Msm} gene and the strain with double deletions of genes rel_{Msm} and *relZ* were constructed on the basis of the WT strain by Sidorov R., the researcher of the Laboratory of Microbial Adaptation, Institute of Ecology and Genetics of Microorganisms RAS (Tkachenko et al., 2021). Strains were stored on Petri dishes with Luria–Bertani (LB) agar medium (Sigma, USA).

Mycobacteria were grown in a test tube with 5 mL of Middlebrook 7H9 liquid medium (HIMEDIA, India) supplemented with glycerol and 25 μg/mL ampicillin (ITW Reagents, USA) and 0.05 % Tween 80 (Rosmedbio, Russia). Cultures (5 mL) grown in tubes for 24 hours in a thermo shaker (37 °C, 200 rpm) were inoculated into 30 mL flasks with a fresh medium and cultivated under the same conditions to an optical density of 2.0–2.4.

Sliding motility. Middlebrook 7H9 medium without glycerol was solidified with 0.3 % agarose (Helikon, Russia). Polyamines and antibiotics were added to 3 mL of sterile medium that was preliminary cooled to 47 °C and dispensed per plate (40-millimeters diameter). Plates were allowed to stand at room temperature for 24 hours prior to inoculation and then 0.5 μL of liquid bacterial culture with optical density 0.2 were inoculated on the surface of the medium in the center of the plate. The cell spread area in the medium surface during growth for the indicated period of time was evaluated after plate incubation at 37 °C in a humidified box.

Measuring of the area of the sliding colony. Sliding colonies grown in plates were photographed on an Olympus C-3040 ZOOM camera (Olympus, Japan). The area was measured in pixels on photos and processed by means of the free trial version of Photoshop CC 2015.5 (Adobe, USA) as compared with the real area. The real area of one pixel was determined by comparing the diameter of the Petri plate in pixels with that of the real plate measured in millimeters.

Measuring of the optical density of the colony. The method had been described in detail earlier (Tkachenko et al., 2021). Photos of the colonies were desaturated using Photoshop CC 2015.5 to determine the optical density of the colony. The colony was singled out using the 'quick selection tool'. 'Brightness' was evaluated using the 'histogram' tool. For illumination inversion, background brightness was taken into account. Based on the obtained background brightness values, the arithmetic mean was calculated and subtracted from the colony brightness.

Determination of the minimum inhibitory concentration of the antibiotic (MIC). MIC was determined by the method of serial twofold dilutions in immunological plates (Minimed, Russia). MIC was taken as the minimal concentration at which there was no visible growth of the cell culture in the well of the plate.

Isolation of GPLs and TLC. Cells were cultured for 48 hours to achieve the stationary phase. Optical density was brought to 1.5 (600 nm). The cells were then washed from the medium and incubated in 0.6 mL of chloroform/methanol (2:1 v/v) at 56 °C for 2 hours in a water bath sonicator (ELMA, Germany). After centrifugation (12,000 rpm, 15 min), the supernatant was purified by extraction with 600 μL distilled water. The organic phase was extracted and evaporated. Lipids were dissolved in chloroform/methanol (9:1); 10 μL were spotted on an aluminum backed silica gel 60 TLC plate (Merck, Germany), and chromatographed with 7 mL of chloroform/methanol (9:1). The TLC plate was soaked briefly in 10 % H_2SO_4 in ethanol and then heated to 180 °C for 90 seconds to visualize lipids.

Biofilm formation. Mycobacterial biofilms were cultured for 48 hours in plastic plates (40 millimeters) (Medpolymer, Russia). The plates contained 4.5 mL Middlebrook 7H9 medium without Tween 80. Cells were washed from Tween 80 and 500 μL were added to plates. Results were photographed.

Phase-contrast microscopy. Cells on the surface of the growth medium were visualized with a phase-contrast tool FATEK 6-7 (LOMO, Russia) and microscope MICMED-6 (LOMO). Results were photographed on the camera of an MC 6.3 microscope (LOMO).

Statistical processing of results. The results were statistically processed using the Statistica 7.0 standard software package (StatSoft Inc., USA). On the graphs, the medians (4–10 experiments) are represented, the vertical segments indicate the values of the first and third quartiles. The statistical significance of differences was assessed using the Mann–Whitney test. Differences were considered significant at $p \leq 0.05$.

Results

Influence of gene activity on the sliding motility of mycobacteria

We showed that all investigated strains were able to slide and form a monolayer of cells on agar surfaces (Fig. 1). At the same time, the control WT strain without deletions formed a colony, the area of which was smaller than that of the strain with the deletion of the rel_{Msm} gene, but differed from the strain with the double $\Delta rel_{Msm}\Delta relZ$ deletion.

The study of the colony edges using phase contrast microscopy confirmed the first conclusion made on the basis of a comparison of the areas of the colonies. Cells of the strains with gene deletions were packed less densely compared to the parental strain (Fig. 2). It indicates that deletion strains are able to slide better than the parent strain.

Concentration of glycopeptidolipids (GPLs) in the cell walls of mycobacteria showed that the parental strain contained the highest amount of GPLs (Fig. 3, *a*). The mutant strains showed the decrease in GPLs concentrations in direct proportion to the increase in the number of deletions. However, a decrease in the amount of GPLs in the cell walls of the deletion strains didn't lead to a decrease in the area of sliding colonies (see Fig. 1). These data may indicate that either GPLs aren't involved in a formation of

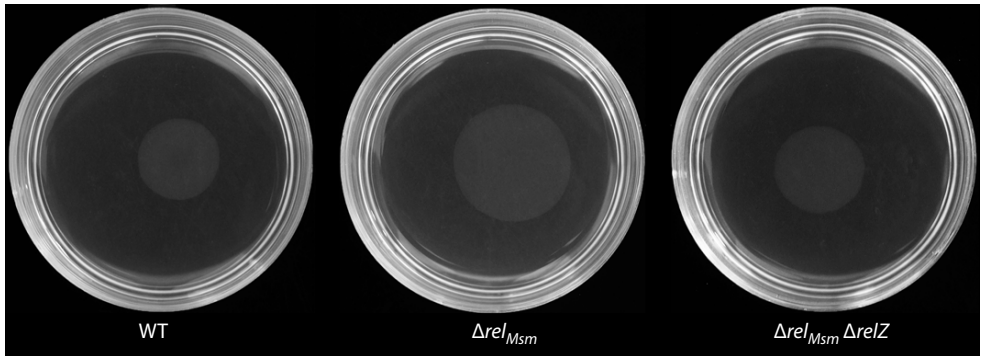

Fig. 1. Sliding motility of mycobacteria strains.

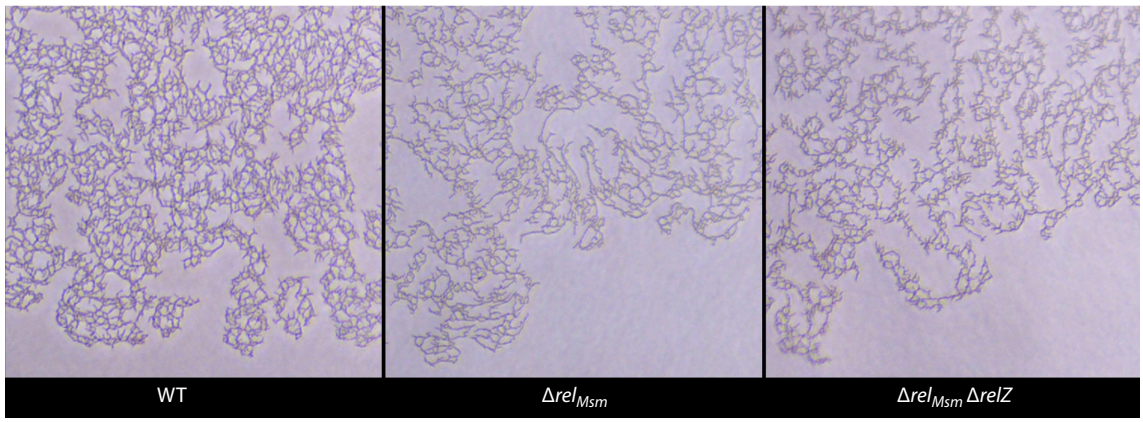

Fig. 2. Edges of monolayers formed by sliding *M. smegmatis* cells of WT and deletion strains.

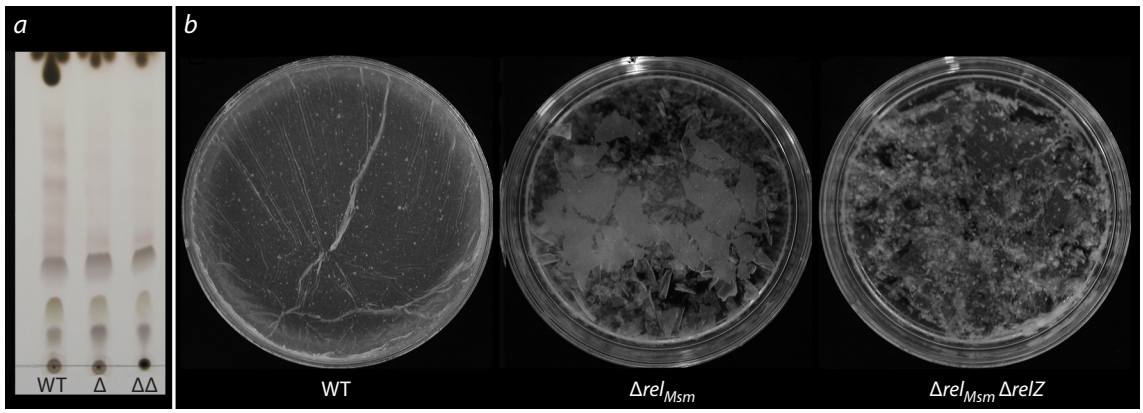

Fig. 3. Dependency between hydrophobicity of cells and GPLs concentration in mycobacterial cell walls.

a, Effect of the *rel*$_{Msm}$ and *relZ* genes activity on the concentration of GPLs in *M. smegmatis* cells; *b*, influence of *rel*$_{Msm}$ and *relZ* genes activity on *M. smegmatis* biofilm formation. Δ, Strain with a deletion of the *rel*$_{Msm}$ gene; ΔΔ, strain with double deletion of the *rel*$_{Msm}$ and *relZ* genes.

hydrophobic surface for sliding or those may not be the only regulators of sliding motility in mycobacteria.

Concentrations of GPLs were not only comparable with our previous results on biofilm formation in mycobacteria (Tkachenko et al., 2021), but also consistent with the information on interdependency between GPLs concentration in the cell wall and hydrophobicity of the bacterial surfaces (Recht et al., 2000). In accordance to the sliding model of mycobacteria, GPLs can form a hydrophobic cell surface, which allows a successful cell sliding through hydrophilic

media. Our studies showed (see Fig. 3, *b*) that the cells of the control WT strain included a high concentration of GPLs in their surface structures and so were able to form biofilms which could hold on the water surface, sinking to the bottom only if their integrity was broken.

In contrast, the strain with one rel_{Msm} deletion contained less GPLs and in addition to defects in biofilm formation associated with impaired activity of the alarmone synthetase gene was characterized by an ability to form the biofilm fragments that were less hydrophobic and so partially fell to the bottom of the plate. The strain with the double deletion rel_{Msm} and *relZ* had the least concentration of GPLs in the cell wall and the lowest hydrophobicity of the surface. Fragments of its biofilms weren't retained on the surface and completely sank to the bottom of the plate (see Fig. 3, *b*). Measurement of the biomass of surface biofilms has showed no statistically significant differences between the strains (Tkachenko et al., 2021). This phase distribution of biofilm fragments was primarily dependent on the hydrophobicity of the cells.

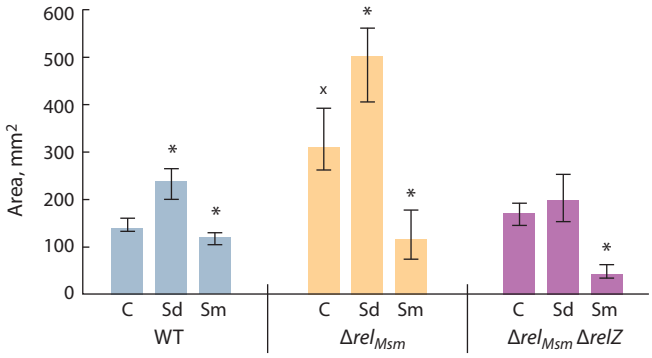

Fig. 4. Effect of polyamines on the sliding of *M. smegmatis* strains.

C – control without polyamine, Sd – spermidine 2 mM, Sm – spermine 2 mM.

* Statistically significant difference from the control colony of the same strain grown on the medium without the addition of polyamines (Mann–Whitney test, $p \le 0.05$).

[x] Statistically significant difference from the control colony of the strain without gene deletions (WT) grown on the medium without addition of polyamines (Mann–Whitney test, $p \le 0.05$).

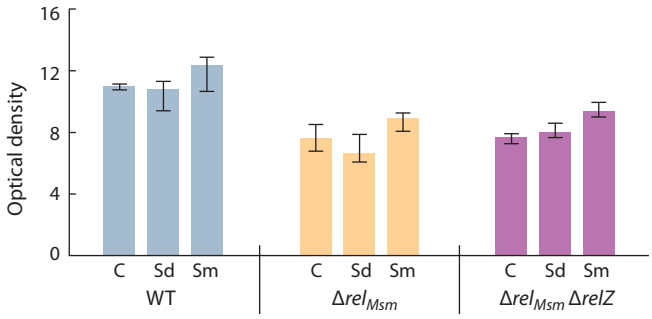

Fig. 5. Effect of polyamines on the optical density of *M. smegmatis* sliding colonies.

C – control without polyamine, Sd – spermidine 2 mM, Sm – spermine 2 mM.

Biogenic polyamines influence on the sliding motility of mycobacteria

Investigation of the sliding motility of the WT strain as compared to the deletion mutants showed that diameters of the sliding colonies of Δrel_{Msm} strain demonstrated statistically significant exceedance of those for WT control strain. However, the areas of colonies formed by the double deletion strain didn't produce statistically significant exceedance of the areas of colonies over the control strain (Fig. 4). Biogenic polyamines spermidine and spermine, when added into the sliding medium, caused different effects. Spermidine increased the areas of colonies in the control strain, as well as in the strain with one deletion, while spermine, inversely, significantly reduced the area of sliding colonies. The polyamine effects were directly proportional to the number of deletions in the strains (see Fig. 4).

Both polyamines, spermidine and spermine, are known to have positive charges due to the presence of amino and imino groups in their molecules that are protonated at the physiological pH values (Gugliucci, 2004). However, they caused a multidirectional effect on sliding. Therefore, the effect of polyamines cannot possibly be explained by their effect on the surface charge of the cell. The decrease in the area of sliding colonies in response to addition of spermine to the cells would be interpreted as a possible bacteriostatic effect. However, as we had shown previously, the used concentrations of polyamines had no effect on the growth rate and viability of mycobacteria in a liquid medium (Tsyganov et al., 2017).

In order to obtain more information about the effect of polyamines on the mass of sliding colonies, we tried to estimate the optical density or the number of cells in a culture. However, due to the hydrophobicity of the surface of mycobacteria grown on the medium without Tween 80, it wasn't possible to completely separate the cells from each other, as well as to separate them from the remains of the agar medium. Therefore, an indirect assessment of the colony mass was carried out by measuring the brightness of the colonies on the shots. As a result, the optical density was standardized relative to the background values of the density of the medium surrounding the colony (Fig. 5). According to results of the measurements, it was found that the change in the areas of sliding colonies is a consequence of the polyamine effect on the sliding motility only, not on cell survival (see Fig. 5).

A statistically significant change in the areas of colonies in the presence of polyamines (see Fig. 4) didn't change the optical density and, respectively, the mass of sliding colonies (see Fig. 5). These data support the conclusion that the polyamines spermidine and spermine had no bactericidal or bacteriostatic effects. Differences in the values of optical density between the parent strain and the strains with gene deletions are a result of concomitant changes in the growth parameters caused by changes in the genotype of the mutant *M. smegmatis* strains relatively to the control WT strain.

Polyamines are interfering in the sliding processes occurring in the presence of antibiotics

To investigate the effects of antibiotics on the sliding mycobacteria, we selected sublethal antibiotic concentrations, which significantly reduced the area of sliding colonies. For comparative analysis of antibiotics, all of the concentrations used were expressed as the multiplicities of the minimal inhibitory concentrations (MIC) values for the antibiotics used, which were previously determined.

When comparing the effectiveness of antibiotics, we have found that rifampicin most contributed to the reduction in the area of sliding colonies of all three strains of mycobacteria, while the streptomycin and isoniazid had approximately the same efficiency (Fig. 6–8). DMNP was shown to have the least antibacterial effect on growing sliding colonies, which is a consequence of its activity primarily for the stationary phase cells (Tkachenko et al., 2021).

In addition, as the DMNP was solute in methanol, we investigated the effect of methanol on the sliding motility of mycobacteria. It was shown that methanol, when added to the medium in the same volume as DMNP (50 μL), had a stimulatory effect on sliding motility as compared to the control (at the absence of methanol) (see Fig. 6–8). Spermine, as well as rifampicin and streptomycin in their minimal concentrations, had a similar inhibition activity as each of these polyamines without antibiotics. The areas of colonies were smaller than the control ones grown on the medium with antibiotic but without polyamine (see Fig. 6).

The polyamine effects changed significantly at their maximal concentrations. The sliding colonies grown on the medium with DMNP addition, but in the absence of polyamines were larger than those grown on the medium supplemented with spermidine and spermine. The combined effect of DMNP with each of these polyamines increased the inhibitory effect on the sliding area to the levels close to those of antibiotics streptomycin and isoniazid in the absence of PA. At the same time, the areas of colonies grown on the medium with the addition of polyamines and streptomycin or isoniazid at their maximal concentrations were more than the area of the control colonies grown in the absence of both PAs. These data can be explained by the protective properties of these polyamines. Therefore the inhibitory effect of DMNP with polyamines on the area of sliding colonies was maximal as compared to that for such antibiotics as streptomycin or isoniazid. Rifampicin was the strongest contributor to the reduction in the areas of colonies of the WT strain without deletions, despite the fact that in the presence of spermidine, the effectiveness of the maximal concentrations of the antibiotic also decreased (see Fig. 6).

Similar results were observed for the strain with rel_{Msm} deletion (see Fig. 7). The most antibacterial effect was de-

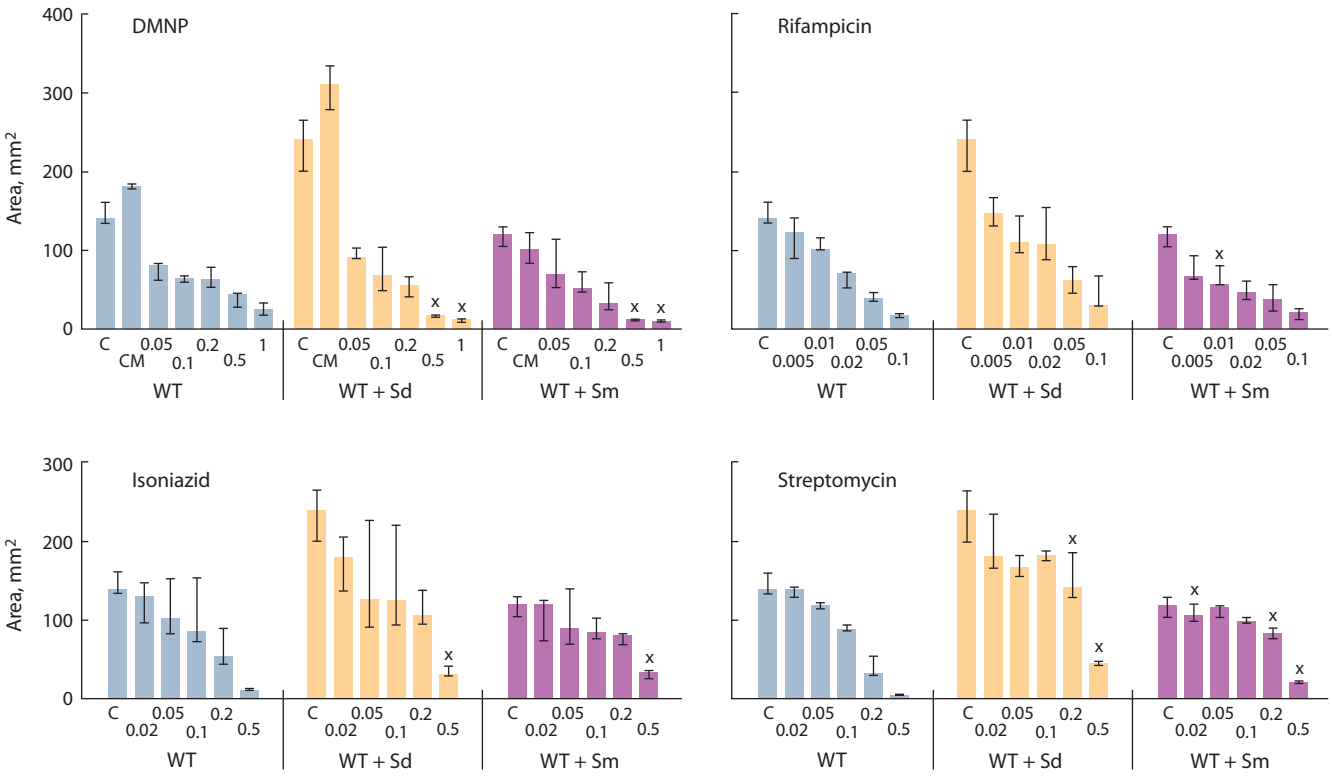

Fig. 6. The action of polyamines and antibiotics on sliding motility of *M. smegmatis* control strain.

Here and in the Figures 7 and 8:
CM – control supplemented with 50 μL of methanol, Sd – spermidine 2 mM, Sm –spermine 2 mM.
[x] Statistically significant difference from a similar control colony without PA (Mann–Whitney test, $p \leq 0.05$).

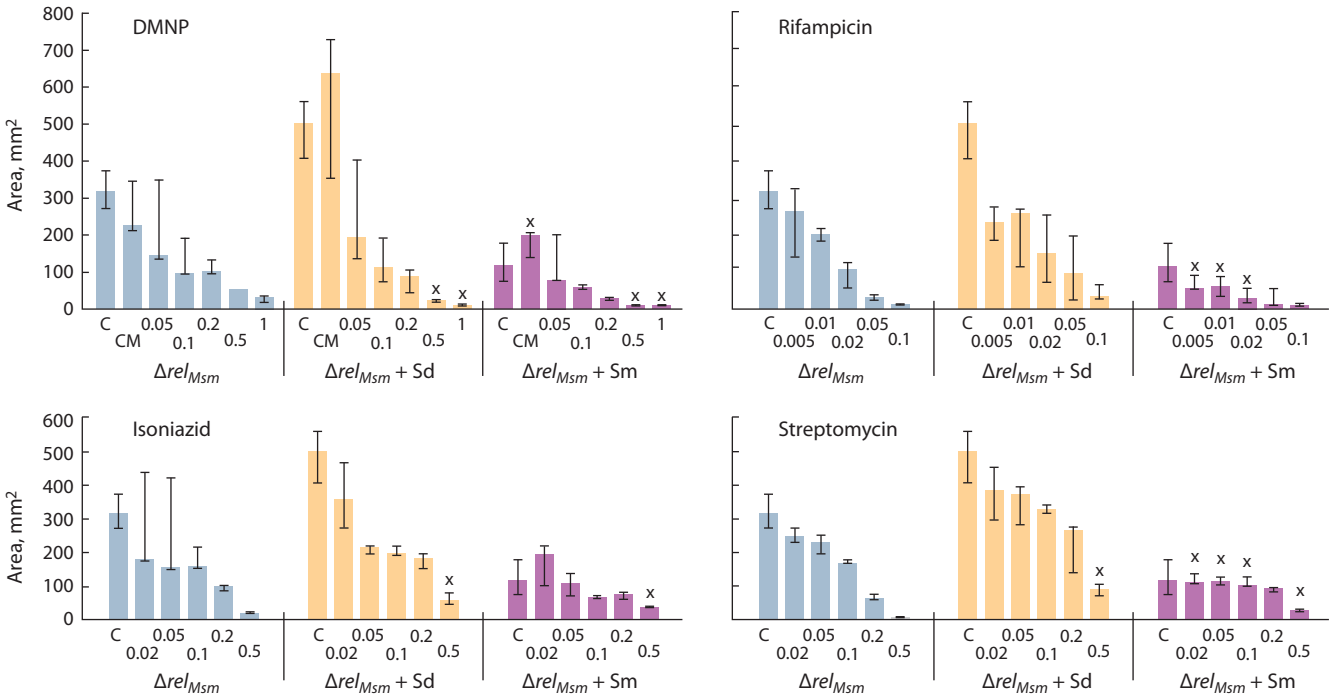

Fig. 7. Effects of polyamines and antibiotics on sliding motility of *M. smegmatis* with rel_{Msm} deletion strain.

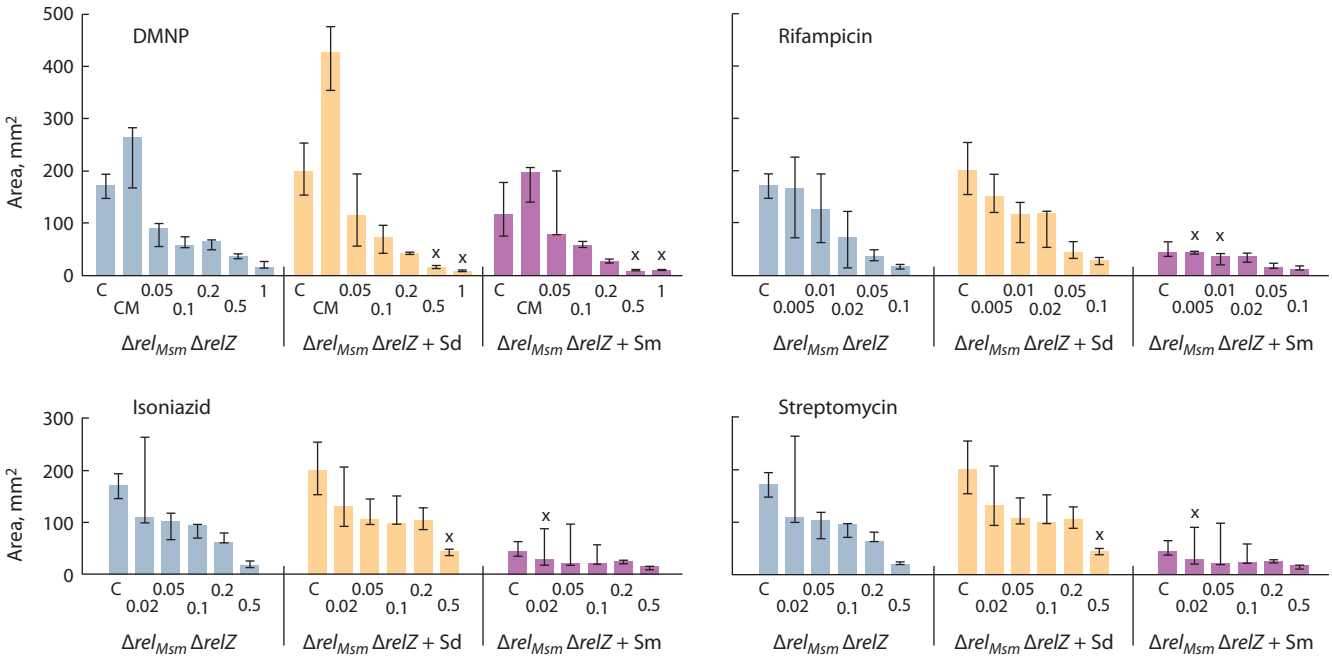

Fig. 8. Effects of polyamines and antibiotics on sliding motility of double deletion *M. smegmatis* Δrel_{Msm} Δ*relZ*.

monstrated by rifampicin. Spermine significantly limited the area of sliding colonies, enhancing the antibacterial effect of minimal streptomycin concentrations and rifampicin. The DMNP activity was increased in the presence of both of these polyamines and, therefore, was greater than the antibacterial effect of isoniazid and streptomycin at their maximal concentrations (see Fig. 7).

Effect of spermidine and antibiotics on the strain with a double deletion of rel_{Msm} and *relZ* genes was similar for that of the other two strains (see Fig. 8). At the same time,

spermine reduced the sliding of mycobacteria at minimal concentrations of isoniazid, streptomycin, and rifampicin. The antibiotic DMNP with both polyamines reduced the areas of sliding colonies more strongly, but on the medium without spermine or spermidine the effectiveness of DMNP was lower than that of other antibiotics.

Discussion

The main factors of sliding are surfactants localized in bacterial cell walls or released into the external environment. Previously, it was considered that the main ones are GPLs, which are localized in the cell wall and are necessary for mycobacteria to slide on the surfaces. However, the results of our investigation on *M. smegmatis* strains deficient in cell wall GPLs (see Fig. 3, *b*) have shown that the colonies formed by the GPLs defective strains exceed the area of the colonies of the parent strain by 1.5–2 times or do not differ from them in area size. The specificity of our study is that the experimental strains with deletions did not stop synthesizing GPLs completely. Despite this, the hydrophobicity of the cell surface of the deletion strains was lower than that of the control strain, which is indirectly confirmed by the results of our studies on the nature of defects in biofilm formation in the deletion *M. smegmatis* strains (see Fig. 3, *b*). Our results suggest that GPLs are not the only regulators of mycobacterial sliding. Therefore, to determine the complete mechanism of the sliding motility, further studies are needed to investigate the role of other lipids that are the components of *M. smegmatis* cell wall and participate in the sliding process.

The multidirectional effect of various polyamines on the diameter of sliding colonies can't be explained only by their influence on the electronegativity of the cell surface, since polyamines have a positive charge. At the same time, the decrease in the areas of colonies caused by spermine is also not a realization of the antibacterial effect and isn't accompanied by a change in colony mass. This suggests that polyamines are able to modulate sliding motility by regulating intracellular processes, possibly acting as a signaling molecule, or directly through changes in cell wall composition. The determination of the sliding mechanism needs to be further investigated.

The combined effects of polyamines and antibiotics showed that rifampicin is the most effective drug against actively dividing cells in the sliding colony. DMNP showed the least activity against sliding colonies on the medium without polyamines. However, in the presence of 2 mM spermidine or spermine, the antibiotic effect was enhanced regardless of the strain of mycobacteria and exceeded that of streptomycin or isoniazid under similar conditions. Polyamines showed a protective effect at maximal concentrations of streptomycin and isoniazid.

The protective function of polyamines was previously known (Sarkar et al., 1995). Nevertheless, the sliding motility in the presence of spermine at minimal concentrations of rifampicin and streptomycin hasn't been previously observed. The stimulatory effect of polyamines in the presence of DMNP provides this antibiotic with an advantage over a range of previously used drugs, since polyamines are widely distributed among the cells and tissues of multicellular organisms and therefore would increase the effectiveness of antibacterial drugs.

Conclusion

In this investigation, we found that the biogenic polyamines spermidine and spermine are able to modulate the sliding motility in mycobacteria and demonstrate a multidirectional effect on this process. Spermine inhibited sliding motility at minimal concentrations of streptomycin and rifampicin. At the same time, both polyamines studied here enhanced the effect of DMNP on the diameter of colonies, making this antibiotic more effective than streptomycin and isoniazid under similar conditions. It has been shown that glycopeptidolipids, apparently, are not the only regulators of mycobacteria sliding. Therefore, the study of sliding mechanisms and the basis of polyamine effects on this process has to be further investigated.

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Nonspecific response of Lake Baikal phytoplankton to anthropogenic impact

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Abstract. In this study, we present the first results on oxidation stress in Lake Baikal phytoplankton and its adaptation to environmental changes under anthropogenic impact. As was shown, the changing of the dominant species of phytoplankton collected from the surface water layer (~0.3 m) took place from February to June 2021. Phytoplankton were collected at a nearshore station (a littoral station at a distance of ~0.01 km from the shoreline, depth to bottom is ~5 m) and an offshore station (a pelagic station at a distance of ~1 km from the shoreline, depth to bottom is ~543 m). In February, dinoflagellates were dominant (~40 %) as well as diatoms (≤33 %) and green algae (≤12 %). Their biomass was 100 mg·m^{-3}. In March, chrysophytes were dominant (up to 50 %) as well as cryptophytes (≤43 %) and dinoflagellates (≤30 %). Their biomass was 160–270 mg·m^{-3}. In April, biomass increased up to 700–3100 mg·m^{-3} with the dominance of large cell dinoflagellates (up to 99 %), chrysophytes (up to 50 %), and cryptophytes (up to 35 %). By the end of the first decade of May, the percentage of dinoflagellates decreased and that of cryptophytes increased. In the second decade of May, the percentage of diatoms increased up to ~26–38 % but phytoplankton biomass was minimal (13–30 mg·m^{-3}). By June, the percentage of diatoms in the samples reached 44–75 % at 60–550 mg·m^{-3}. The oxidation stress of phytoplankton as a nonspecific adaptive response to a prolonged, intensive, or recurrent effect of a stress factor was estimated from the content of thiobarbituric acid reactive substances (TBARS). The mean content of these substances (markers of the lipid peroxidation) was determined spectrophotometrically. The oxidation stress of phytoplankton was revealed only when diatom algae dominated. It can be explained by adaptation of algae of other classes to the stress factor. The content of the lipid peroxidation markers in the coastal phytoplankton collected close to the settlement of Listvyanka known as a large touristic center was estimated from 100 to 500 μg·g^{-1} of dry weight of sample. During the period of diatom blooming in 2016 and 2018, oxidation stress of phytoplankton collected near large settlements was found. In phytoplankton from deep-water pelagic stations most remote from settlements, stress was not revealed. Using the method of gas chromatography, we showed a lower (up to 15 %) content of polyunsaturated fatty acids in phytoplankton characterized by stress occurrence. This confirms cell membrane damages. In Lake Baikal surface water, we found a higher content of synthetic anionic surfactants (sodium alkylbenzene sulfonates), which are components of detergents and cause oxidation stress of hydrobionts (up to 30 ± 4 μg·L^{-1}). The presence of these substances in a water ecosystem can result in exhausting of phytoplankton cell resources, homeostasis imbalance, stress, pathological changes, and rearrangements in phytoplankton assemblage.
Key words: Baikal; phytoplankton oxidation stress; stress response in diatoms; alkylbenzene sulfonates; TBARs, adaptation of phytoplankton.

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Неспецифическая адаптационная реакция байкальского фитопланктона в ответ на антропогенную нагрузку

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Аннотация. Представлены первые результаты по изучению окислительного стресса фитопланктона из озера Байкал и его адаптивных свойств к изменению среды обитания в условиях повышенной антропогенной нагрузки. Анализ фитопланктона, отобранного в поверхностном слое воды (~0.3 м) на прибрежной (глубина 5 м, расстояние от берега 10 м) и пелагической станциях (глубина 543 м, расстояние от берега 1000 м), показал смену доминирующих видов с февраля по июнь 2021 г. В феврале доминировали динофитовые (~40 %), диатомовые (до 33 %) и зеленые (до 12 %) водоросли с низкой биомассой – 100 мг/м^3. В марте преобладали золотистые (до 50 %), криптофитовые (до 40 %) и динофитовые (до 30 %) (биомасса 160–270 мг/м^3). В апреле наблюдалось увеличение биомассы до 700–3100 мг/м^3 с доминированием крупноклеточных динофитовых (до 99 %), золотистых

(до 50 %) и криптофитовых (до 35 %) водорослей. К концу первой декады мая доля динофитовых снизилась и увеличилась доля золотистых. Уровень развития диатомовых повышался во второй декаде мая до ~26–38 % при минимальной биомассе фитопланктона (13–30 мг/м^3). К июню доля диатомовых в пробах достигала 44–75 % при биомассе 60–550 мг/м^3. Окислительный стресс фитопланктона как неспецифическую адаптационную реакцию на длительное, интенсивное либо повторяющееся воздействие стресс-фактора оценивали по содержанию в пробах веществ, вступающих в реакцию с тиобарбитуровой кислотой. Среднее содержание данных веществ – маркеров перекисного окисления липидов – было оценено спектрофотометрически. Окислительный стресс фитопланктона выявлен только в период доминирования в пробах диатомовых водорослей. Это может быть объяснено лучшей адаптацией водорослей других отделов к стресс-фактору. Содержание маркеров перекисного окисления липидов в прибрежном фитопланктоне, отобранном вблизи населенного пункта и крупного туристического центра пос. Листвянка, составило от 100 до 500 мкг/г сухой массы пробы. В 2016 и 2018 гг. в период массового развития диатомовых водорослей обнаружен окислительный стресс фитопланктона вблизи крупных населенных пунктов. В фитопланктоне глубоководных пелагических станций, максимально удаленных от населенных пунктов, стресс не найден. С помощью метода газовой хроматографии показано более низкое (до 15 %) содержание полиненасыщенных жирных кислот в планктоне, характеризующемся наличием стресса. Это свидетельствует о повреждении мембран клеток. Повышенное содержание анионных синтетических поверхностно-активных веществ, а именно алкилбензолсульфонатов натрия, являющихся компонентами моющих средств и вызывающих окислительный стресс гидробионтов, обнаружено в поверхностной воде оз. Байкал (до 30 ± 4 мкг/дм^3). Наличие данных соединений в водной экосистеме может приводить к исчерпанию ресурсов фитопланктона, нарушению гомеостаза, стрессу, патологическим изменениям и перестройкам планктонного сообщества.
Ключевые слова: Байкал; окислительный стресс фитопланктона; стресс диатомовых; алкилбензолсульфонаты; тиобарбитуровая кислота; адаптационная реакция фитопланктона.

Introduction

Under the effect of external factors on a living cell and the organism as a whole, a complex of nonspecific and specific adaptive defense responses occurs. Nonspecific responses also called stress are the response of a living system to intense or unusual irritants. This allows assessing the scale of the impact of stress factors on an organism. In this case, adaptation mechanism includes the activation of all systems of an organism counteracting stress factors and supporting homeostasis and dynamic balance between the organism and the environment. Rate of exposure to chemicals as a cell stress factor varies depending on their characteristics (concentration, physical and chemical properties of the molecules) as well as on the individual response and the adaptation potential. In the case of prolonged, repeated, or intense exposure, a malfunction of the organism's adaptive reactions may occur. This leads to resource depletion, homeostasis imbalance, distress, and pathologies (Poryadin, 2009).

Pollution of aquatic ecosystems by xenobiotics and absence of adaptation of water dwellers to their effect are an acute problem for the 21st century. Some of the most common persistent micropollutants in aquatic ecosystems are polycyclic aromatic hydrocarbons (PAH) (Vega-López et al., 2013) and heavy metals (Srivastava et al., 2006). Alkylbenzene sulfonates are the most common persistent macropollutants (Lewis, 1991; Jorgensen, Christoffersen, 2000). The common feature of the substances mentioned above is their ability to induce oxidative stress and hypoxia of the cell and organism as a whole.

The increasing activity of the enzymes such as superoxide dismutase, catalase, and glutathione peroxidase due to initial or minor stress is an indicator of the oxidation stress, which is a nonspecific adaptive response. On the contrary, prolonged or intense exposure to a stress factor may result in suppressing of the effects of enzyme activity of a living organism, disease, and death. The aldehydes including malone dialdehyde being formed due to the destruction of the lipids of the cell membranes by reactive oxygen species (lipid peroxidation) is another indicator of oxidation stress (Marnett et al., 1999; Hampel et al., 2008; Goncalves et al., 2017; Zhou et al., 2018; Nikonova et al., 2022).

Aquatic microorganisms such as phytoplankton are capable of activating the defense systems of the organism such as the hormonal system, adenine nucleotide exchange system, prostaglandin and antioxidant systems. The latter is better investigated. It usually allows resistance to natural physical and chemical factor effects but can not cope with xenobiotic impact (Karthikeyan et al., 2013). Phytoplankton are extremely sensitive to environmental changes. The state of the entire ecosystem depends on their well-being.

Particular attention should be paid to diatoms, which are good indicators of water quality. Diatoms are used in the biomonitoring of heavy metals and organic pollutants such as petroleum, polyaromatic hydrocarbons (PAH), pesticides, polychlorinated biphenyls (PCB), and anionic surfactants (Datta et al., 2019). This is due to diatoms being considered the most diverse phytoplanktonic group in all aquatic ecosystems. Many of them are common to water bodies of different types and live all over the world. This allows comparing the data of the analysis. Rapid diatom response to both short-term and long-term physical and chemical environmental changes is noticed (Dixit et al., 1992). Different sensitivity of various species of diatoms is known (Datta et al., 2019). For example, some of them are susceptible to eutrophication (*Eunotia* sp., *Diatoma vulgare*, *Gomphoneis herculeana*, *Achnanthidium* sp., *Achnanthes subhudsonis* var. *kraeuselii*), effects of organic pollutants (*Nitzschia palea*, *Nitzschia fonticola*), heavy metals (*Fragilaria capucina*, *Achnanthidium minutissimum*), electroconductivity fluctuations (*Fragilaria ulna* var. *acus* (Kütz.) Lange-Bert.), pH changes (*Eunotia* sp., *Pinnularia* sp., *Eunotia exigua*, *Gomphonema angustum*, *Amphora veneta*, *Gomphonema rautenbachiae*), flow rate (*Melosira* sp., *Cocconeis* sp.), mass transport and sedimentation (*Navicula* sp.,

Nitzschia sp., *Surirella* sp.), concentration of nutrients such as nitrogen (*Gomphonema parvulum*, *Eolimna minima*, *Nitzschia palea*) and phosphorous (*Gomphoneis herculeana*, *Achnanthidium* sp., *Achnanthes subhudsonis* var. *kraeuselii*, *Luticola goeppertiana*, *Navicula recens*, *Nitzschia inconspicua*, *Nitzschia palea*, *Rhopalodia* sp., *Eunotia* sp.), and others.

Lake Baikal is the deepest and oldest rift lake containing 23,615.39 km^3 of ultra fresh water with a total mineralization of 96–98 mg · L^{-1}. Due to basin peculiarities, the surface area of Lake Baikal is 32,822 km^2, of which the littoral zone occupies only ~3.4 %. The littoral contains maximal biodiversity (more than 98 % of species) with biomass up to ~100–620 kg per hectare at depths of <4–70 m. Phytoplankton inhabit the littoral and the pelagic zones down to ~750 m of depth but its maximal abundance characterizes the photic zone at depths of ~60–120 m and the zone of the intensive vertical water mixing by wind at depths up to ~200–300 m. In the spring season, phytoplankton primary production reaches ~160 tones per hectare (Votintsev et al., 1975; Nikonova et al., 2022). About 200 species of planktonic algae were registered in the water column of the lake. More than 50 of them are diatom species (Votintsev et al., 1975; Rusinek, 2012). The percentage of diatoms reaches 50–90 % of the total phytoplankton biomass (Popovskaya et al., 2015). The littoral is much more exposed to negative anthropogenic impact than the pelagic zone. Since 2000, changes in nearshore phytoplankton have already been observed (Bondarenko, Logacheva, 2017). In 2019, the oxidation stress of the coastal phytoplankton was described (Nikonova et al., 2022) but the reasons behind this phenomenon have not yet been established unambiguously.

The objectives of our study were to evaluate the nonspecific adaptive response of Lake Baikal phytoplankton to anthropogenic impact and to assess the possibility of using them as a bioindicator.

Materials and methods

Water sampling for determining the phytoplankton composition. All samples were collected from stations of three types: nearshore stations (depth to bottom up to 30 m); short-distance pelagic station (distance from the shoreline ~1–3 km); central pelagic stations (distance ~10–30 km both from the east and west shorelines).

Sampling was carried out in 2021 during the under-the-ice period from the third decade of February to the first decade of May and during the open water period from the third decade of May to the first decade of June. Water from the surface down to a depth of 0.5 m was sampled regularly at the stationary stations to analyze the phytoplankton composition. The nearshore stationary station is characterized by the depth to bottom ~5 m and the minimal distance from the shoreline of 10 m. The short-distance pelagic station is characterized by the depth 543 m and the distance from the shoreline of 1000 m. The stationary stations are situated opposite the Sennaya River mouth in Listvennichnyi Bay located in the southern basin of the lake. Besides, phytoplankton were sampled in three basins of Lake Baikal in 2016 and 2018. Water samples of 1 L volume were poured into bottles and fixed with Lugol's solution. Then, samples underwent concentration according to the classical method by cell sedimentation during 10 days at room temperature in the dark (Nakashizuka, Stork, 2002). The concentrates were used to assess species composition, number of cells, and biomass.

Net sample collection. The representative samples of phytoplankton biomass were obtained using the Juday-type net with 100 μm mesh size. Live phytoplankton collected at the stationary sampling sites were transported to the laboratory in thermoses and filtered through the cellulose acetate filters (0.45 μm, Vladisart, Russia) using the filter-apparatus (Duran Group, Germany). The lipid peroxidation markers were analyzed without delay. The residual biomass was wrapped in aluminum foil and stored at −70 °C. The samples of phytoplankton biomass, which could not be transported to the laboratory as live biomass, were filtered, frozen at −20 °C, transported to the laboratory, and stored at −70 °C.

Microscopy and estimation of phytoplankton qualitative characteristics. Cells in concentrated sedimentation samples were subsequently identified using a light microscope Amplival (Carl Zeiss, Jena) at ×800 magnification. Species diversity was estimated according to conceptual guides for measuring species diversity (Matvienko, Litvienko, 1977; Starmach, 1985; Round et al., 1990; Tsarenko, 1990; Glezer et al., 2011). Cell number in each sample of 0.1 mL volume (N, cells·10^3/mL) was counted according to formula

$$N = \frac{\overline{N} \cdot 10 \cdot V_2 \cdot 1000}{V_1 + V_2},$$

where $\overline{N}$ is average cell number per volume, V_1 – volume of decanted water, V_2 – volume of the concentrated sample.

The cell number was converted into the cell biomass (B) taking into account the individual shape, size, and volume of the cell of every species (Makarova, Pichkily, 1970; Belykh et al., 2011).

Water sampling for determining the concentration of anionic surfactants. The surface water of 0.1 L volume was collected from Lake Baikal at depths up to 0.5 m from 30 May to 18 June 2021 using the bathometer to analyze anionic surfactant concentrations. Water samples were placed into dark glass bottles and fixed with ethyl alcohol (1 mL). To analyze anionic surfactant and phytoplankton composition both samples were collected at the same time from 30 March to 18 April 2021. Water was also sampled in the mouths of rivers Sennaya, Bannyui Ruchei, Krestovka, Bolshaya Cheremshanka, and Malaya Cheremshanka, inflowing into Lake Baikal. Samples were filtered through micro-cellulose acetate filters (0.45 μm, Vladisart, Russia) using the filter-apparatus. After that, the filter with suspended matter was cut and put into the 10 mL glass flask. To extract the anionic surfactants, 1 mL of distilled ethyl alcohol was added to each sample. Then, samples underwent extraction for 5 min using a 50 Hz ultrasonic bath. After that, the extracts were placed in plastic 2-mL Eppendorf taste-tubes and centrifuged at 13,000 rpm for 3 min. The supernatant was merged with the filtered water, and the obtained samples were stored at +3 °C until the analysis.

Identification of alkylbenzene sulfonates in water samples. The identification of alkylbenzene sulfonate homologues in water sample extracts concentrated onto DSC-18 reversed-phase sorbent (0.5 g, Supelco, USA) was carried out with a reversed-phase high performance liquid chromatograph Milichrom A-02 (Eco-Nova, Russia) coupled to a UV-detector. A water solution of linear alkylbenzene sulphonate (LAS)

mixture (GSO 8578-2004) was used as an external standard (100 mg/mL, Analytic-Chim, Russia). Chromatography was performed at 60 °C using 2 × 75 mm Nucleosil 100-5-C_{18} column (Eco-Nova, Russia). The characteristics were the following: solvent A – water with 0.1 % (v/v) trifluoroacetic acid (TFA); solvent B – acetonitrile (ACN) with 0.1 % (v/v) TFA; isocratic – 40 % B in 0.3 mL; then gradient – 40–100 % B in 2 mL; injection volume – 100 μL; detection – UV 224 and 230 nm.

The determination of anionic surfactants. Spectrophotometric qualitative analysis of anionic surfactants in water samples collected in Lake Baikal and its tributaries was implemented using methylene blue. The samples of 50 mL volume were extracted with chlorophorm according to previous work (Nikonova et al., 2022). A double beam UV-Vis Cintra-20 spectrophotometer (GBC, Australia) with a Czerny–Turner configuration monochromator and holographic diffraction grating provided precision and accuracy of the obtained data. Standard quartz cuvettes of 1 cm path length were used. Absorbance was measured at 651.5 nm.

Qualitative and quantitative analyses of fatty acids. To extract fatty acids (FA), 1.2 mL of Folch solution (chloroform–methanol, 2:1 by volume) was added to each sample, then it was placed into an ultrasonic bath (3 × 5 min) (Nikonova et al., 2020, 2022). After that, 0.35 mL of distilled water was added to the extracts (chloroform–methanol–water partition 2:1:1 v/v). The mixtures were vigorously shaken and centrifuged at 3,000 rpm for 3 min. The supernatant was put into glass vials and the solvent was evaporated using an argon stream. Then, 4.5 mL of 2 % H_2SO_4 solution in methanol was added to the dry fraction. Esterification of fatty acids was carried out at 55 °C during 1.5 h. Fatty acid methyl esters (FAMEs) were extracted with *n*-hexane (3 mL × 2 × 2 min). The extracts were dried with anhydrous Na_2SO_4. The internal standard (1 mg·mL^{-1} of do-decyl ether solution in *n*-hexane) was added to the extract. The sum analysis of both free and etherified FAs by gas chromatography coupled to mass-spectrometry as well as by gas chromatography coupled to flame ionization was carried out using the "6890B GC System, 7000C GC/MS Triple Quad" (Agilent, USA) and "GC-2010 Plus" (Shimadzu, Japan) with "Optima-17MS" 30 m × 0.25 mm columns (Macherey-Nagel, Germany).

Estimating the oxidation stress of phytoplankton. To estimate the oxidation stress of phytoplankton, we analyzed thiobarbituric acid (TBA) reactive substances (TBARS). To prepare the samples of 0.15–0.20 g of weight, we used the analytical method described earlier (Haraguchi et al., 1997; Al-Rashed et al., 2016) with our modifications (Nikonova et al., 2022). The analysis was carried out with a Cintra-20 spectrophotometer.

Results

Twenty genera of microalgae including 39 taxa of phytoplanktonic algae and 21 taxa of benthic algae collected both at the nearshore station (depth to bottom ~5 m, distance from the shoreline 10 m) and at the pelagic one (depth to bottom 543 m, distance from the shoreline 1000 m) were identified from February 23 to May 26 in 2021. Among phytoplanktonic algae, 7 classes were identified: chrysophytes (5 taxa), blue-green algae (3 taxa), cryptophytes (4 taxa), dinoflagellates (7 taxa), diatoms (11 taxa), green algae (8 taxa), and euglenophytes (1 taxon). The total number of the samples was 23.

In the nearshore zone, phytoplanktonic algae biomass varied significantly from 13.4 to 3111 mg · m^{-3}, and the dominant species of phytoplankton changed in the period mentioned above. Dinoflagellates *Gymnodinium baicalense* and *Peridinium baicalense* (~40 %), diatoms *Synedra acus* subsp. *radians* (up to 33 %), and green algae *Monoraphidium arcuatum* (up to 12 %) were dominant in February. Their total biomass (102 mg · m^{-3}) and cell number (100 · 10^3 cells · L^{-1}) were small. Changes in phytoplankton composition with the dominance of chrysophytes *Dinobryon cylindricum* (25–50 %), cryptophytes *Rhodomonas pusilla* (15–36 %), and dinoflagellates (~30 %) were fixed in March. Their biomass reached 160–270 mg · m^{-3} and their cell number was (130–170) · 10^3 cells · L^{-1}.

The maximal phytoplankton biomass (3110 mg·m^{-3}) and the maximal cell number (1030 · 10^3 cells·L^{-1}) were registered in April. Dinoflagellates *G. baicalense* and *P. baicalense* (92–99 %) dominated till the third decade of April. The maximal biomass was recorded before the end of the first decade of May (930 mg·m^{-3}) and was defined by chrysophytes (~63 %), cryptophytes (~18 %), and diatoms (~16 %).

By the end of the third decade of May, the maximal biomass decreased down to minimal values (13–30 mg·m^{-3}). A decrease in phytoplankton growth and changes in the phytoplankton assemblage structure were fixed. Diatoms (30–40 %) and cryptophytes (20–30 %) were the dominants in contrast to chrysophytes (13–20 %), dinoflagellates (~15 %), and green algae (6–16 %).

In the pelagic zone, the qualitative characteristics of the phytoplankton were low at the end of March (N = 35 · 10^3 cells·L^{-1}, B = 93 mg·m^{-3}). Dinoflagellates (up to 50 %), cryptophytes (up to 30 %), and chrysophytes (up to 20 %) prevailed. By the end of the first decade of April, dinoflagellates *G. baicalense* and *P. baicalense* (up to 90 %) dominated (N = 100 · 10^3 cells·L^{-1}, B = 900 mg · m^{-3}). In the middle of April, the dominants were the same but the cell number reached 200·10^3 cells·L^{-1} and the biomass increased up to 1600 mg · m^{-3}.

In the first decade of June, diatoms dominated at the stationary stations (up to 80 %). The detailed composition of Lake Baikal phytoplankton collected in Listvennichnyi Bay during the diatom bloom period is given in Table 1.

The increase in quantitative characteristics of phytoplankton is shown in Figure 1. These are the percentages of the total biomass, cell number, and the biomass of diatoms of Lake Baikal phytoplankton collected in Listvennichnyi Bay during the spring season of 2021.

We did not find lipid peroxidation products (LPOP) in net samples of phytoplankton collected in March–April before the intense diatom bloom. During the period of intense diatom blooming LPOP were not revealed in four pelagic samples, and in two other samples their contents were minimal (13 and 50 μg·g^{-1} of dry weight (d. w.)). LPOP content in nearshore phytoplankton was estimated in a range from 120 to 630 μg · g^{-1} d. w. The samples of nearshore phytoplankton were collected at two sample sites: (1) the stationary station in front of the River Sennaya and (2) the station in front of the settlement of Listvyanka. Two independent net samples

Table 1. Composition of Baikal phytoplankton sampled in Listvennichnyi Bay (June 5, 2021) and its quantitative characteristics such as cell number (N · 10^3 cells · L^{-1}) and biomass B (mg · m^{-3})

Taxa	Sampling stations							
	1		2		3		4	
	N	*B*	*N*	*B*	*N*	*B*	*N*	*B*
Chrysophyta								
Dinobryon cylindricum	8.3	16	23	44	0.8	1.52	7.2	14
Chrysochromulina parva	2.7	0.14	24	1.2	7.0	0.35	2.9	0.15
Cystes of Chrysophyta	2.4	1.2	12.2	6.1	11.2	5.6	1.5	0.75
Chrysosphaera melosira	–	–	–	–	–	–	0.16	0.02
The sum	13	17	60	51	19	7.5	12	15
Cryptophyta								
Rhodomonas pusilla	2.7	0.59	12	2.7	8.4	1.8	5.1	1.12
Cryptomonas sp. 1	5.1	14	14	40	1.7	4.8	0.96	2.7
Cryptomonas sp. 2	0.08	0.20	0.75	1.9	–	–	0.06	0.15
The sum	7.9	15.1	27	45	10	6.6	6.1	4.0
Dinophyta								
Gyrodinium helveticum	0.53	11	0.6	12	0.56	11.2	0.40	8.0
Peridinium baicalense	–	–	–	–	0.16	5.6	–	–
Glenodinium sp. 1	0.75	1.13	0.6	0.9	1.12	1.68	1.04	1.6
Glenodinium sp. 2	–	–	0.3	1.1	–	–	0.40	1.4
The sum	1.28	12	1.5	14	1.8	18.5	1.8	11
Bacillariophyta								
Aulacoseira baikalensis	–	–	–	–	–	–	0.04	0.6
Aulacoseira islandica	2.0	9.2	11	48	0.32	1.47	0.88	4.1
A. islandica spores	0.15	0.6	0.45	1.8	–	–	0.28	1.12
Synedra acus subsp. *radians*	3.2	6.1	33	63	11.2	21	3.6	6.8
Synedra ulna var. *danica*	–	–	–	–	–	–	0.08	0.26
Synedra ulna	–	–	–	–	–	–	0.04	0.09
Nitzschia graciliformis	2.1	0.53	2.1	0.53	0.32	0.08	0.12	0.03
Cyclotella minuta	0.75	1.58	1.1	2.31	0.48	1.0	0.64	1.34
Cyclotella baicalensis	0.23	3.9	0.08	1.36	0.08	1.36	0.04	0.68
Stephanodiscus meyeri	29	15	397	199	3.1	1.56	155	78
Stephanodiscus sp.	0.08	0.04	–	–	–	–	0.40	0.20
Asterionella formosa	0.08	0.05	–	–	–	–	–	–
The sum	38	37	444	316	16	27	161	93
Chlorophyta								
Monoraphidium arcuatum	0.75	0.21	12	3.4	2.8	0.78	2.2	0.62
Monoraphidium contortum	0.08	0.01	–	–	–	–	–	–
Chlamydomonas sp.	–	–	1.05	0.47	–	–	–	–
The sum	0.8	0.2	13	3.9	2.8	0.8	2.2	0.6
Euglenophyta								
Euglena sp.	–	–	0.08	0.26	–	–	–	–
The sum	–	–	0.08	0.26	–	–	–	–
The sum of all genera	61	81	546	429	49	60	183	123

Note. The coastal stations are marked by Nos. 1–3 and the pelagic one is marked by No. 4.

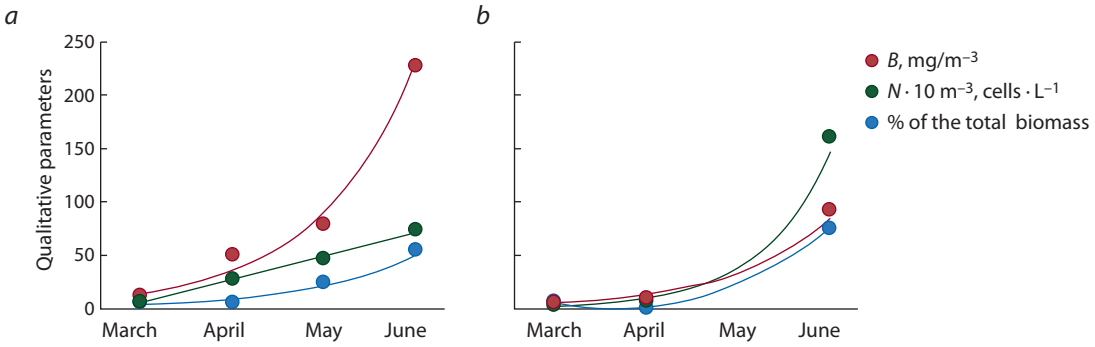

Fig. 1. The increase in quantitative characteristics of phytoplankton in 2021: *a*, nearshore phytoplankton; *b*, pelagic phytoplankton.

Table 2. LPOP content (μg·g^{-1} of dry weight) in phytoplankton sampled from the stationary stations

Sample Nos., $m = 6$	Sampling number (n)									Average
	1			2			3			
	Measuring number									
	x_1	x_2	x_3	x_1	x_2	x_3	x_1	x_2	x_3	
Pelagic station in 1 km from the shoreline in front of the River Sennaya										
1	0	0	0	140	149	158	0	0	0	50 ± 6
2	28	39	49	0	0	0	0	0	0	12.9 ± 1.7
Nearshore station No. 1 in front of the River Sennaya (1 km from settlement of Listvyanka)										
3	67	85	85	298	328	282	328	351	359	240 ± 30
4	122	142	136	60	41	60	185	163	177	121 ± 16
Nearshore station No. 2 in front of the River Malaya Cheremshanka (settlement of Listvyanka)										
5	884	884	884	323	350	403	643	656	669	630 ± 80
6	379	379	379	139	165	148	1096	1106	1106	540 ± 70

were collected at each site. Three sub-samples were picked from each sample excluding a step of biomass homogenization to estimate the distributional heterogeneity of the analyzed substances. The increase in the level of phytoplankton oxidation stress was fixed at the sample site No. 1. The content of LPOP reached 120–240 μg · g^{-1} in biomass collected from the site No. 1 and in biomass collected from the site No. 2 it was 540–630 μg · g^{-1} (Table 2).

The diatom *S. acus* subsp. *radians* prevailed (92–95 %) in phytoplankton samples ($m = 20$) collected in three basins of Lake Baikal during the first decade of June in 2016, 2018 (Fig. 2). Diatoms of other species as well as chrysophytes contribute ≤5 % to the total biomass. This allowed us to compare the characteristics of the samples taken from different sites. The pelagic sample stations were located in the center of the lake ($m = 3$). Among the nearshore stations, background stations ($m = 4$) as well as the sites located near the cities and large settlements ($m = 9$) were chosen. The samples of the axenic laboratory culture of *S. acus* subsp. *radians* were also analyzed ($m = 3$) (Table 3). In nearshore phytoplankton, LPOP content as a marker of oxidation stress was estimated in a range from 14 to 340 μg · g^{-1} d. w. and it was not found in the biomass of pelagic phytoplankton collected from the central stations.

Anionic surfactants were found in all of the water samples. The qualitative composition was represented by homologues of sodium alkylbenzenesulfonate (Fig. 3). The concentration of these pollutants in water of the nearshore zone close to large settlements achieved 21 ± 3 μg·L^{-1}. It was less than 10 μg ·L^{-1} in water of the background stations and less than 5 μg ·L^{-1} in pelagic water. Anionic surfactants concentrations in water of Lake Baikal tributaries such as the Rivers Bolshaya Cheremshanka (12.6 ± 1.5 μg ·L^{-1}), Malaya Cheremshanka (8.1 ± 1.0), Krestovka (74.5 ± 9.0), Bannyi Ruchei (14.8 ± 1.8), Sennaya (30.1 ± 3.7) were found in a wide range. The Krestovka River flows through the Listvyanka settlement and is characterized by maximal discharge and surfactants concentrations in water.

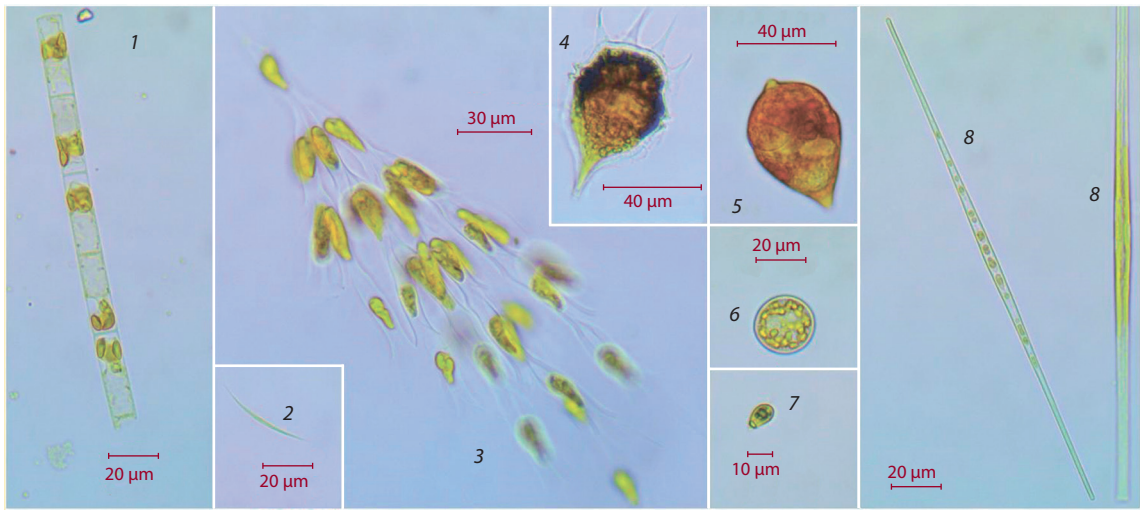

Fig. 2. Spring phytoplankton of Lake Baikal collected in Listvennichnyi Bay: *1*, *A. islandica*, *2*, *Kolliella longista*, *3*, *D. cylindricum*, *4*, *Peridinium baicalense*, *5*, *G. helveticum*, *6*, *C. minuta*, *7*, *Rh. pusilla*, *8*, *S. acus* subsp. *radians*.

The photos were obtained with the use of a LOMO Micromed-6 light microscope at ×400 magnification.

Table 3. Contents of the unsaturated fatty acids and oxidation stress marker substances in phytoplankton with the diatoms as a dominant

Sample station	Year	Lake zone	ΣFA, mg·g^{-1}	UFA, %	LPOP, µg·g^{-1}
Marituy River – Solzan River	2016	Pelagic	16	72	0
Ludar Cape – Frolikha River	2016		17	72	0
Elokhin Cape – Davsha settlement	2016		24	70	0
Average				71	0
Aya Bay	2016	Nearshore distanced from settlements	28	66	0
Ludar Cape	2016		49	68	0
Shamanka Bay	2016		29	60	14
Elokhin Cape	2016		18	55	14
Average				62	7
Kultuk settlement	2016	Nearshore not far from large settlements	24	56	80
	2018		21	56	100
Baikalsk town	2016		27	58	280
	2016		27	58	340
Baikalsk pulp and paper mill region	2016		26	60	160
	2018		21	54	164
In front of the mouth of Tyya River where the Severobaikalsk city wastewater inflows	2016		27	46	100
	2018		16	58	11
Senogda Bay, 8 km from the Tyya River mouth	2016		14	40	190
Average				54	158
S. acus axenic laboratory culture			35	74	0

Note. The limit of LPOP determination (LOD) of 0.5 µmol·mL^{-1} via spectrophotometry was determined by Rakita et al. (2020); UFA – unsaturated fatty acids, which means the sum of all monounsaturated and polyunsaturated fatty acids.

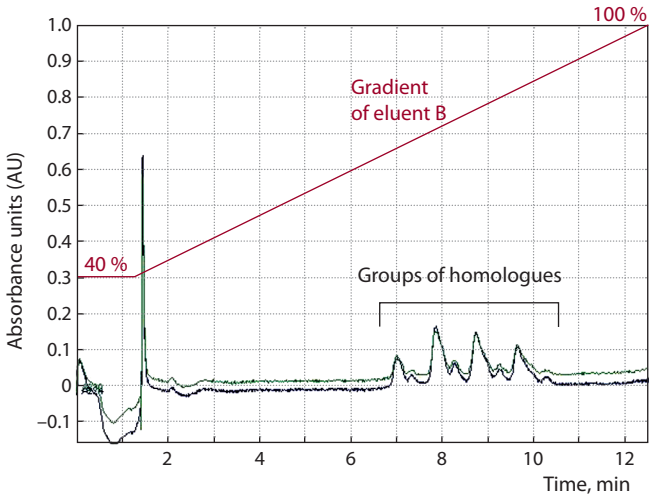

Fig. 3. The chromatogram of alkylbenzene sulfonates homologues in water from Listvennichnyi Bay (April, 2022).

Peaks identification was carried out according to FR.1.38.2017.27043 method of sodium alkylbenzene sulfonate (sulfanol) determination by HPLC-UV (in Russian).

Discussion

For Lake Baikal, marine peculiarities of climate as well as a delay of seasonal onsets in the coastal zone are common compared to the nearby continental zone (Ladeishchikov, 1987). That is why June is a spring month at the Lake Baikal territory. Analysis of phytoplankton collected at stationary sites during the spring season (March–June 2021) shows clear changes in dominant species. Diatom abundance increased from 5 % in March–April to 44–75 % in the first decade of June. This event has been noticed earlier and is typical for Baikal (Vorobyeva, 2018).

High contents of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) characterize the planktonic assemblage with the *Synedra acus* subsp. *radians* as a dominant. The major fatty acids (FAs) are C18:3-ω-3 (~7–9 %) and C20:5-ω-3 (~10–23 %, average 17 %). C20:4-ω-6 and C22:6-ω-3 FAs are presented in less content (Nikonova et al., 2020). The mentioned FAs are the most destructible under the free radical effect. Polyunsaturated FAs are mostly concentrated in lipid bilayer of the cell membranes. The membrane of *S. acus* is covered with a silica cell wall and contains ~30 % of PUFA, which makes it vulnerable to free radical attack.

There are two known routes for lipid destruction in the cell. The first is the α-, β-, and ω-oxidation of lipids by enzymes with the formation of numerous vital compounds. The second is the lipid peroxidation. The final products of peroxidation are peroxides and aldehydes including malondialdehyde (MDA). Peroxidation of unsaturated FAs takes place in the case of free radical attack of reactive hydrogen atoms of the methylene group of the alkyl chain. These groups should be conjugated to a pair of the C–C double bonds. The lipid peroxyl radical, which formed as a result of the mentioned process, then reacts with another fatty acid to produce a new lipid radical and lipid hydroperoxide; thus, this chain reaction continues (Fig. 4).

Nonspecific adaptation response of the cell and the organism as a whole is the response to a stress factor effect, which is common for different organisms. This response aims to restore the homeostasis of the system. An example of a nonspecific response is oxidation stress. For instance, the oxidation stress of green algae due to UV-B (Al-Rashed et al., 2016) and the oxidation stress of aquatic plants as a result of heavy metals impact (Srivastava et al., 2006) were described. The oxidation stress of Lake Baikal phytoplankton during the intense diatom bloom found by us is an unspecific adaptation response to environmental changes. Nevertheless, the data of the analysis of phytoplankton with chrysophytes, cryptophytes, dinoflagellates as dominant species show the absence of oxidation stress markers. This is related to cell membrane structure of the mentioned algae, which contains cellulose and hemicellulose. It makes the membrane more resistant to free radical impact and enables a better adaptation of these microalgae.

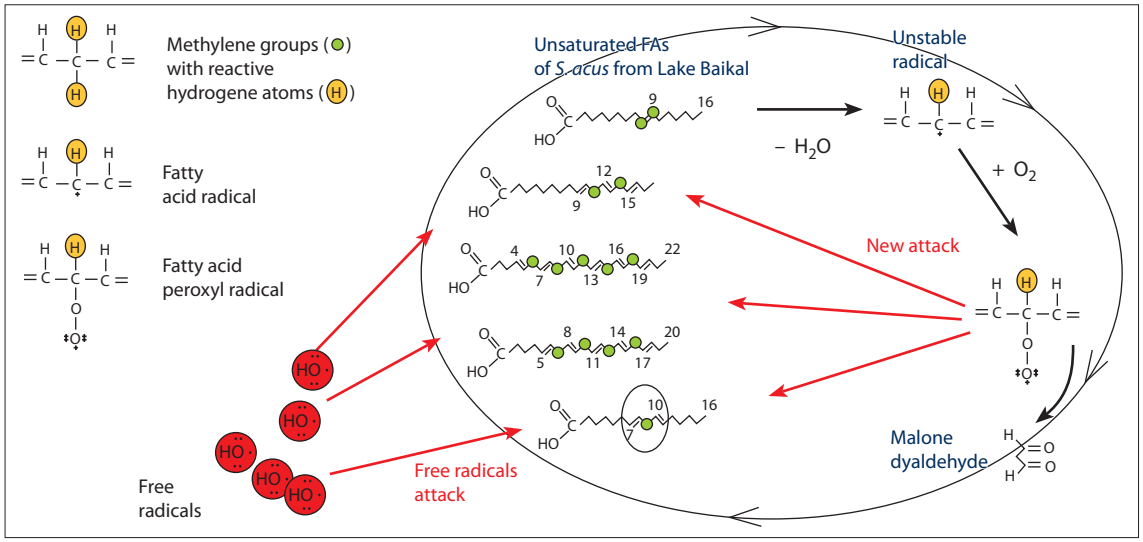

Fig. 4. Free radical mechanism of preliminary unsaturated FAs peroxydation of Lake Baikal phytoplankton with the *S. acus* dominance.

The significant inhomogeneity of the results of LPOP determination was noticed when collecting two samples at each station. The relative standard deviation reached 90 %. Because of the high reactivity of free MDA, the results of determination of this substance content in the biological samples are usually understated. This marks the lipid peroxidation process, which is taking place in a cell at the moment (Zelzer et al., 2013). Inhomogeneity of the results is most probably induced by the high reactivity of MDA. So, the LPOP determination results evidently can not provide the precise qualitative characteristic of the stress. Though the LPOP occurrence unambiguously confirms the diatom cell membrane potential exhaustion, malfunction of the adaptive reaction, homeostatic imbalance, and the evident oxidation stress of the phytoplankton collected in the regions of a high intensity anthropogenic load.

The absence of the oxidation stress of the pelagic phytoplankton from the central stations, as well as lower oxidation stress of the phytoplankton collected from the background nearshore stations and the UFA content decrease in stressed phytoplankton confirm the correlation of the stress of *S. acus* with the effects of a stress factor (see Table 3). The last one is unusual for the species mentioned above, and the protective adaptation mechanism has not formed yet.

The authors of this work suggest sodium linear alkylbenzene sulfonates to be a potential stress factor resulting in the lipid peroxidation of Baikal diatoms. The concentrations of these pollutans in surface water achieved critical values up to 30 ± 4 μg · L^{-1} near large settlements and cities in 2019–2021 though. In the only sample concentration reached 54 ± 7 μg · L^{-1} though for the most of samples it does not exceed 10 ± 1.2 μg · L^{-1}. Surfactants of this type possess maximal hazard, and their affect causes acute toxicity to water organisms, as well as chronic influence including oxidation stress at ≤10–20 μg · L^{-1} (Lewis, 1991; Jorgensen, Christoffersen, 2000). Anionic surfactants and alkylbenzene sulfonates in particular were related to hazard substances[1] according to the United Nations Environment Programme (UNEP) and to especially hazard substances[2] for the unique ecosystem of Lake Baikal presented as a UNESCO World Heritage Site.

Conclusion

The oxidation stress of nearshore Baikal phytoplankton with diatoms *Synedra acus* subsp. *radians* as a dominant was revealed in regions of increased anthropogenic load. An assumption that *S. acus* is a susceptible bioindicator to xenobiotic effect causing the oxidation stress is proposed. During the under-the-ice period, the oxidation stress of phytoplankton was not found, which can be explained by the domination of the algae of other classes and their better adaptation to reactive oxygen species effect. We believe the nearshore phytoplankton stress to be caused by local critical concentrations of anionic surfactants in the coastal water of Lake Baikal.

[1] Linear alkylbenzene sulfonates. SIDS Initial Assessment Report for 20th SIAM. UNEP Publications, Paris, France, 19–21 April, 2005.

[2] Order of Russian Federation No. 83 (21.02.2020). On approval of standards of maximum permissible actions on unique ecological system of Lake Baikal and list of hazardous substances including most dangerous substances, high dangerous substances and moderate dangerous substances for unique ecological system of Lake Baikal. The Ministry of Natural Resources and Ecology of Russian Federation.

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Effect of copper ions on the associations of *Azospirillum* bacteria with wheat seedlings (*Triticum aestivum* L.)

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Abstract. The physiological and biochemical activity of plant–microbial associations enables them to determine the mobility, bioavailability, and accumulation of heavy metals in plant tissues. These abilities are the basis for the use of plants and their associated microorganisms in the development of approaches that ensure both the prevention of the ingress of toxic metals into food crops and the extraction of pollutants from polluted soils by using phytoremediation technologies. Whether plant–microbial complexes are used successfully depends on the knowledge of how specific organisms interact with heavy metals. We evaluated the effect of copper ions on common wheat (*Triticum aestivum* L.) inoculated with three plant-growth-promoting rhizobacteria (PGPR) of the genus *Azospirillum*. We analyzed the growth variables of 14-day-old wheat seedlings, the content of photosynthesis pigments, the activity of plant oxidoreductases, and the accumulation of copper by plant tissues. All strains more or less compensated for copper toxicity to seedling development and increased metal accumulation in roots and shoots. Copper affected the photosynthetic apparatus of the inoculated plants, primarily by decreasing the content of chlorophyll *b*. An analysis of the activity of plant oxidoreductases (peroxidases and phenoloxidases), which are involved in the physiological responses of plants to pollutant stress, showed strain-specific dependence and a significant effect of copper on the inoculated plants. Overall, the obtained results clearly show that the effect of *Azospirillum* on the physiological and biochemical status of wheat is diverse. The compensatory effect of bacteria on copper toxicity and the simultaneous increase in metal accumulation in plant tissues can be considered as mutually exclusive crop-production aspects associated with the growing of food plants in heavy-metal-polluted areas.
Key words: *Azospirillum*; *Triticum aestivum*; copper; seedlings; photosynthetic pigments; peroxidase; laccase; tyrosinase.

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Влияние ионов меди на ассоциации бактерий рода *Azospirillum* с проростками пшеницы (*Triticum aestivum* L.)

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Аннотация. Растительно-микробные ассоциации в результате своей физиолого-биохимической активности способны определять подвижность, биодоступность и накопление в растительных тканях тяжелых металлов. Указанные способности являются основой для использования растений и ассоциированных с ними микроорганизмов в разработке подходов, обеспечивающих как предотвращение попадания токсичных металлов в пищевые культуры, так и извлечение поллютантов из загрязненных земель с помощью технологий фиторемедиации. Успешное применение растительно-микробных комплексов в той или иной области зависит от изученности механизмов взаимодействий в системе конкретных организмов с тяжелыми металлами. Целью представленных исследований была оценка влияния ионов меди на эффекты бактеризации растений пшеницы мягкой (*Triticum aestivum* L.) тремя штаммами *Azospirillum*, обладающими свойствами стимуляции роста растений (PGPR). В ходе эксперимента анализировали ростовые параметры 14-суточных проростков пшеницы, содержание пигментов фотосинтеза, активность растительных оксидоредуктаз и аккумуляцию металла растительными тканями. Все штаммы в той или иной степени компенсировали фитотоксическое воздействие меди на развитие проростков и увеличивали ее аккумуляцию в корнях и побегах. Показано отчетливое усиление воздействия меди на фотосинтетический аппарат бактеризованных растений, выражающееся в изменении содержания основных пигментов, в первую очередь уменьшении хлорофилла *b*. Анализ активности растительных оксидоредуктаз (пероксидаз и фенолоксидаз) как участников физиологических ответов растений на стрессовые воздействия выявил их штаммоспецифичный характер и существенное влияние меди на бактеризованные растения. В целом полученные

результаты показали отчетливое разноплановое влияние исследованных штаммов азоспирилл на физиолого-биохимический статус растений пшеницы. Выявленный компенсаторный эффект бактерий на фитотоксическое воздействие меди и одновременно повышение ее накопления в растительных тканях могут рассматриваться как взаимоисключающие аспекты растениеводства, связанные с выращиванием пищевых растений на загрязненных тяжелыми металлами площадях.
Ключевые слова: *Azospirillum*; *Triticum aestivum*; медь; проростки; фотосинтетические пигменты; пероксидаза; лакказа; тирозиназа.

Introduction

Soil pollution by heavy metals is a serious environmental problem. The accumulation of heavy metals in ecosystems leads to their increased uptake by plants and migration along food chains up to humans (Larionov M.V., Larionov N.V., 2010). Plant–microbial complexes are most important for the transformation, translocation, and accumulation of heavy metals in nature. The physiological and biochemical activity of microorganisms and plants enable them to transform heavy metal compounds and determine metal mobility, bioavailability and accumulation (Nadeem et al., 2015). These abilities are the basis for the use of plant–microbial associations in the development of approaches to prevent the input of toxic metals into food crops, on the one hand, and in the development of technologies for cleaning agricultural landscapes from pollutants (phytoremediation), on the other hand. Whether plant–microbial complexes are used successfully depends on the knowledge of how specific organisms interact with heavy metals.

The vital activity of *Azospirillum* bacteria, typical members of the associative microflora of plants, is closely linked to the root system of plants, mainly that of cereals (Reis et al., 2015). Azospirilla are facultative diazotrophs that can fix atmospheric nitrogen under microaerophilic conditions and produce phytohormones (auxins, gibberellins, and cytokinins) and other phytoactive substances. This makes them prominent plant-growth-promoting rhizobacteria (PGPR) (Bashan, De-Bashan, 2010; Fukami et al., 2018). Azospirilla use different strategies to colonize plant roots, which enables differentiation between epiphytic strains (those able to colonize only the root surface) and endophytic strains (those able to penetrate into the root interior) (Rothballer et al., 2003). By interacting with plants, azospirilla promote their growth and reduce environmental stress through various mechanisms, including increased mobilization and absorption of minerals (Bashan, De-Bashan, 2010). A typical associative plant for azospirilla is wheat. Inoculation with *Azospirillum* is beneficial to agriculturally important crops, including wheat (Teixeira Filho et al., 2017; Galindo et al., 2019; Boleta et al., 2020).

Members of the species *A. brasilense* are resistant to a number of toxic metals (Co, Cu, Zn, and Cd). Endophytic and epiphytic azospirilla differ markedly in their resistance to metals (Kamnev et al., 2005, 2007).

Copper is a very important trace element involved in various plant physiological processes, such as electron transport during photosynthesis, mitochondrial respiration, response to oxidative stress, and hormonal signaling. As a cofactor, this metal is part of many plant enzymes and proteins, such as superoxide dismutase, cytochrome *c* oxidase, amino oxidase, laccase, tyrosinase, polyphenol oxidase, and plastocyanin (Yruela, 2005; Pichhode, Nikhil, 2015). However, high concentrations of copper are phytotoxic and cause various kinds of damage to plants, including wheat (Quartacci et al., 2000; Michaud et al., 2007; Dang et al., 2009). Inoculation with *A. brasilense* increases wheat resistance to stress caused by the presence of Cu^{2+} ions (El-Samad, 2017). Yet, inoculation with strains that use different strategies to interact with plants may differ in its effect, which requires additional study. Previous studies (Kamnev et al., 2007) showed that epiphytic and endophytic *A. brasilense* strains differ in the mechanisms of metal resistance, which is linked to the accumulation of poly-3-hydroxybutyrate as a factor contributing to survival under adverse conditions. On the basis of those results, we assumed that under heavy metal stress, such strains may differ in their interaction with plants.

We examined the effect of copper ions on soft wheat (*Triticum aestivum* L.) inoculated with different plant-growth-promoting strains of *A. brasilense*.

Materials and methods

Azospirillum brasilense Sp7 (IBPPM 150), *A. brasilense* Cd (IBPPM 288), and *A. baldaniorum* Sp245 [IBPPM 219, formerly *A. brasilense* Sp245 and reclassified by dos Santos Ferreira et al. (2020)], from the IBPPM RAS Collection of Rhizosphere Microorganisms (http://collection.ibppm.ru) were used in this study.

Bacteria were grown in a liquid or on an agarized (1.5 %) medium composed as follows (g/L): K_2HPO_4 – 0.1; KH_2PO_4 – 0.4; $MgSO_4 \cdot 7H_2O$ – 0.2; NaCl – 0.1; $CaCl_2$ – 0.02; $FeSO_4 \cdot 7H_2O$ – 2.0; NTA-3Na – 5.6; $NaMoO_4 \cdot 2H_2O$ – 0.002; sodium malate, 3.8; NH_4Cl – 1.0; pH 7.0. Copper was used as a copper sulfate at 0.5 mmol/L, which, according to preliminary studies, is the minimal concentration inhibiting bacterial growth.

Seeds of soft spring wheat (*Triticum aestivum* L. cv. Saratovskaya 29) were obtained from the Federal State Budgetary Scientific Organization "Federal Center of Agriculture Research of the South-East Region". After being calibrated, the seeds were washed with a detergent for 10 min with shaking to remove hydrophobic contaminants and were sterilized with 70 % (vol./vol.) ethanol for 3 min, then with diacide (1:1000; 666 mg/L cetylpyridine chloride and 333 mg/L ethanol mercury chloride) for 5 min, then with a mixture of rifampicin (4 μg/mL) and amphotericin B (20 μg/mL) at room temperature for 24 h with shaking (120 rpm), and finally with diacide (1:1000) for 2.5 min. After each stage, the seeds were repeatedly washed with sterile distilled water. The sterilized seeds were placed one by one in sterile biological tubes (20 × 300 mm) containing 15 cm^3 glass beads with a diameter of 2 mm (SiLibeads, Sigmund Lindner,

Warmensteinach, Germany) and 6 mL of Hoagland's solution for plant growth (Hoagland, Arnon, 1950). $CuSO_4 \cdot 5H_2O$ was added to the growth medium to achieve a Cu^{2+} ion concentration of 0.5 mmol/L. The control medium was copper-free. The tubes with experimental plants were inoculated with a microbial suspension.

For inoculation, bacteria were grown in a liquid malate medium for 18 h, after which they were centrifuged (11 000 g, 5 min), washed twice, and resuspended in a sterile medium. Each tube with a 3-day-old seedling was inoculated with 30 µL of bacterial suspension to an inoculant concentration of 10^7 cells per mL in the plant growth solution. Noninoculated plants were used as the control. The plants were grown under controlled conditions at 24 °C for 14 days with a 13/11 h day/night illumination period. Lighting was provided by Fluora fluorescent lamps (Osram, Munich, Germany).

At the end of plant growth, we analyzed the growth variables of 14-day-old seedlings, the content of photosynthesis pigments, the activity of plant oxidoreductases, and the accumulation of copper in plant tissues. The morphological variables (root and shoot length) were measured with a calibrated stainless ruler. The roots and shoots were then dried to a constant weight.

Biochemical analysis of seedlings included the measurement of the content of photosynthetic pigments and the examination of the enzyme activity of roots and shoots. The content of chlorophylls *a* and *b* (Chl *a* and Chl *b*) and carotenoids was determined spectrophotometrically in ethanol leaf extracts, as described earlier (Lyubun et al., 2020).

For determining the activity of plant oxidoreductases (peroxidases, laccases, and tyrosinases), shoots and roots (0.2–0.3 g) were ground in a mortar with quartz sand and were resuspended in 2 mL of 0.2 M Na/K phosphate buffer (pH 6.0). The homogenate was centrifuged at 5000 g for 10 min, the sediment was additionally washed with a phosphate buffer and was recentrifuged. Enzyme activity and protein content were determined in the resultant combined supernatants by using an Evolution 60 spectrometer (Thermo Scientific, USA). The protein content was determined by the Bradford method (Bradford, 1976).

Peroxidase activity (EC 1.11.1.7) was measured by using 23 µM of 2,7-diaminofluorene (DAP) in 0.05 M Na/K-phosphate buffer (pH 6.0) at 600 nm (Criquet et al., 2000); 1 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ammonium (ABTS) in 0.05 M Na-tartrate buffer (pH 3.5) at 436 nm (Yang et al., 2007); and 0.3 mM *o*-dianisidine (DAZ) in 0.05 M Na/K-phosphate buffer (pH 6.0) at 460 nm in the presence of 0.5 mM H_2O_2. Laccase activity (EC 1.10.3.2) was determined by the formation of the oxidation products of 7.5 µM syringaldazine (SGZ) in 0.05 M Na/K-phosphate buffer (pH 6.0) at 525 nm (Leonowicz, 1981) and 23 µM DAP in 0.05 M Na/K-phosphate buffer (pH 6.0) at 600 nm (Criquet et al., 2000). Tyrosinase activity (EC 1.10.3.3) was determined in a 4 mM solution of 3,4-dihydroxyphenyl-L-alanine (DOPA) and 50 mM Tris-HCl (pH 7.5) at 475 nm (Criquet et al., 2000). Enzyme activity was expressed in mmol of oxidized substrate per min per mg of protein.

The total plant content of copper was analyzed with an atomic absorption spectroscopy system equipped with a graphite furnace (Thermo Scientific iCE 3500 Solaar). A 3-mL portion of HNO_3 (Suprapur; Merck, Darmstadt, Germany) and 2 mL of H_2O_2 (30 %; JTBaker Chemical Co., Philipsburg, New Jersey, USA) were added to Teflon containers containing 200 mg of plant material. The samples were then processed in a CEM MARS Xpress microwave digester (Matthews, NC, USA) by using an optimized program. After processing, the volume of the samples was adjusted to 20 mL with ultrapure deionized water and was analyzed for metal content by spectrometry.

All experiments and analyses were carried out in at least three replicates, each replicate using five to eight plants. Means were compared by Student's *t* test ($p \leq 0.05$). Correlation analysis was conducted by using Spearman rank correlations. Microsoft Excel 2007 (Microsoft Office, USA) and Statistica 13.0 (TIBCO Software Inc. 2017, Statsoft Russia) software were used for statistical analysis.

Results

Plant growth

Copper was significantly toxic to seedling development, decreasing both length and biomass of roots and shoots. The inhibition of root growth was more pronounced (root length decreased by 58 % and root weight by 13 %), as compared with shoots, whereas shoot length decreased by a mere 8 % and shoot biomass weight was not changed significantly (Table 1).

The effect of inoculation on seeding development within 14 days under copper-free conditions depended on the strain used (see Table 1). *A. brasilense* Sp7 had a significant effect only on shoot biomass, with an increase of 16 %, as compared with the noninoculated control. *A. baldaniorum* Sp245 significantly reduced root length (by 20 %) and increased root biomass (by 22 %). *A. brasilense* Cd affected the seedlings the most, significantly increasing root and shoot biomass (by 52 and 53 %, respectively).

The effect of inoculation on root and shoot length and biomass was changed by copper. In seedlings inoculated with strain Cd, root biomass tended to decrease and root length decreased by 34 %. The shoot length and biomass of the seedlings inoculated with strain Cd decreased by 14 and 18 %, respectively. The effect of copper on the root and shoot length of the plants inoculated with strains Sp245 and Sp7 was nonsignificant. Yet, strain Sp245 reduced root biomass but increased shoot biomass (by 11 %); by contrast, strain Sp7 increased root biomass by 33 % and slightly reduced shoot biomass.

With all three strains, inoculation reduced copper toxicity to seedlings, which was most evident as increases in root length (by 1.7–2.4 times) and root biomass (by 30–68 %). The negative effect of copper on root length was fully mitigated only with strain Sp7. Inoculation with all strains not only compensated for the effect of copper on root biomass but also significantly increased it relative to the copper-free noninoculated control.

Content of photosynthesis pigments

In noninoculated seedlings, copper had only a slight effect on the content of and ratio between photosynthesis pigments (Table 2).

Inoculation of seedlings grown without copper promoted the content of chlorophylls *a* and *b* and their total amount by

Table 1. Length and dry weight of cv. Saratovskaya 29 seedlings grown in the presence of copper ions and *Azospirillum* strains

Treatment	Roots		Shoots	
	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}
	Length, cm			
Noninoculated	18.8 ± 2.2	7.8 ± 0.9#	38.3 ± 4.5	35.3 ± 6.2
Sp245	14.9 ± 3.7*	14.7 ± 1.7*	31.5 ± 7.9	37.5 ± 6.9
Cd	20.9 ± 1.6	13.7 ± 3.9#*	41.2 ± 1.7	35.2 ± 5.2#
Sp7	18.2 ± 3.6	18.6 ± 1.9*	36.0 ± 4.9	39.1 ± 6.1
	Weight, mg			
Noninoculated	117.0 ± 14.2	101.3 ± 6.6#	227.4 ± 15.7	237.9 ± 15.9
Sp245	142.4 ± 13.6*	132.1 ± 11.7*	242.6 ± 27.1	270.2 ± 26.7#*
Cd	178.1 ± 16.6*	163.7 ± 23.4*	347.3 ± 21.2*	285.5 ± 23.2#*
Sp7	127.5 ± 13.4	169.8 ± 11.4#*	263.4 ± 24.5*	257.1 ± 16.2*

Note. Values represent means ($n \geq 6$) ± standard deviation. Here and in the Tables 2–4: * values differ significantly from noninoculated control, $p \leq 0.05$; # values differ significantly from copper-free treatment, $p \leq 0.05$.

Table 2. Content of photosynthesis pigments in cv. Saratovskaya 29 seedlings grown in the presence of copper ions and *Azospirillum* strains

Treatment	Chl *a*	Chl *b*	Chl *a*+*b*	Chl *a*/Chl *b*	Carotenoids, mg/g
	mg/g				
	Without Cu^{2+}				
Noninoculated	0.99 ± 0.05	0.76 ± 0.05	1.75 ± 0.11	1.31 ± 0.14	0.35 ± 0.04
Sp245	1.10 ± 0.03*	0.82 ± 0.03*	1.91 ± 0.09*	1.34 ± 0.14	0.38 ± 0.29
Cd	1.08 ± 0.12	0.80 ± 0.02	1.88 ± 0.15	1.34 ± 0.12	0.38 ± 0.19
Sp7	1.11 ± 0.09*	0.82 ± 0.11	1.92 ± 0.12*	1.33 ± 0.15	0.38 ± 0.21
	With Cu^{2+}				
Noninoculated	1.09 ± 0.11	0.87 ± 0.08#	1.96 ± 0.22	1.24 ± 0.32	0.36 ± 0.03
Sp245	1.00 ± 0.03#	0.50 ± 0.04*#	1.50 ± 0.12*#	2.00 ± 0.51*#	0.19 ± 0.06*
Cd	0.88 ± 0.02*#	0.34 ± 0.02*#	1.22 ± 0.05*#	2.55 ± 0.12*#	0.15 ± 0.09*#
Sp7	1.06 ± 0.04	0.53 ± 0.06*#	1.60 ± 0.12*#	1.99 ± 0.11*#	0.20 ± 0.02*

Note. Values represent means ($n \geq 6$) ± standard deviation.

11, 8, and 9 %, respectively, with strain Sp245, and by 12, 8, and 10 %, respectively, with strain Sp7. The ratio between chlorophylls *a* and *b* and the carotenoid content changed slightly in response to treatment with strains Sp245 and Sp7.

With strains Sp245, Cd, and Sp7, the effect of copper was manifested in two ways: (1) significant decreases in the content of the pigments, mostly chlorophyll *b* (by 43, 58, and 39 %, respectively); in the total content of chlorophylls *a* and *b* (by 23, 38, and 18 %, respectively); and in the content of carotenoids (by 47, 58, and 44 %, respectively). (2) Significant increases in the Chl *a*/Chl *b* ratio (by 61, 105, and 60 %, respectively).

Accumulation of copper in seedling tissues

The content of copper ions in the tissues of seedlings grown in a copper-free environment varied from 17 to 28 μg/g of dry biomass. The content of copper ions in the seedlings grown in the presence of 0.5 mmol/L of copper is given in the Figure.

In noninoculated seedlings, the accumulation of copper in the roots and shoots was 116 and 59 μg/g, respectively. These variables were strongly increased by inoculation with *Azospirillum*. Treatment with strains Sp245, Cd, and Sp7 increased the accumulation of copper in roots by 6, 10, and 14 times and in shoots by 4, 7, and 9 times, respectively.

Activity of oxidative enzymes of wheat seedlings

The activity of the total peroxidase of wheat plants was measured by using several substrates, which allowed us to take into account different isoforms of this enzyme. In general, according to our data (Table 3), the activity of peroxidase was significantly higher in roots than in shoots.

The presence of copper in the growth solution at the concentration used did not cause a significant change in root peroxidase activity, but peroxidase activity tended to decrease in shoots.

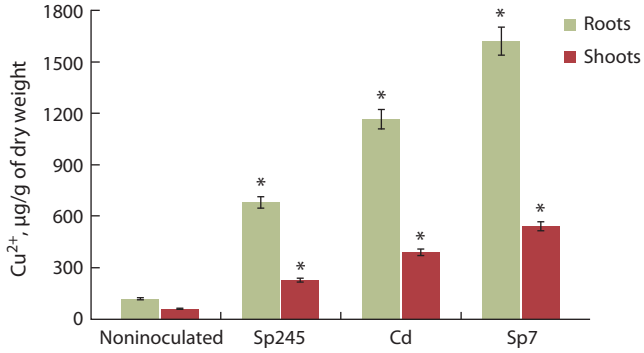
Inoculation of seedlings with strains Sp245 and Cd increased DAZ and ABTS peroxidase activity by more than 1.5 and 5 times, respectively, in roots but not in shoots. With strain Sp7, the enzymatic response to inoculation was different. In roots the activity of DAZ and ABTS peroxidases remained unchanged, whereas in shoots the activity of DAZ, ABTS, and DAP peroxidases increased by 7.0, 5.8, and 7.5 times, respectively. With strains Cd and Sp7, the activity of DAP peroxidase in roots decreased.

In the presence of copper, the peroxidase activity of the wheat seedlings inoculated with strain Sp7 was sharply increased in roots (by 6.3, 5.7, and 7 times) and was less increased in shoots (by 1.6, 1.6 and 2.5 times) for DAZ, ABTS, and DAP, respectively. In the shoots of seedlings inoculated with strains Cd and Sp245, copper increased peroxidase activity by two to three times.

The measured results for the activity of copper-containing plant phenol oxidases (laccase and tyrosinase) are given in Table 4. In noninoculated seedlings, the addition of copper to the growth solution increased laccase activity in roots (by 1.5 times, with DAP), and decreased it in shoots (by 1.75 times, with SGZ).

Depending on the strain used, inoculation significantly changed laccase activity. Strain Cd caused the most significant increase in root laccase activity (by 6 times, with DAP), and strain Sp7 promoted shoot laccase activity (by 7.5 times, with SGZ). Copper reduced the effect of inoculation. With strains Sp245 and Cd, the laccase activity of the roots was comparable to that in noninoculated plants (with SGZ). By contrast, strain Sp7 promoted laccase activity by almost 7 times. In the presence of copper, the laccase activity in the shoots of inoculated seedlings varied depending on the strain and test substrate. The activity increased the most (by 1.8 times, with DAP) with strain Cd and decreased (by 2 times, with SGZ) with strain Sp7.

Similar to laccase activity, tyrosinase activity in the presence of copper increased in roots and decreased in shoots (by 1.7 times in either case). Without copper, strains Sp245 and Cd promoted tyrosinase activity by 1.5 and 5.6 times, respectively, in roots but not in shoots, whereas strain Sp7, on the contrary, promoted tyrosinase by 6.7 times in shoots but not in roots. Copper reduced the effect of inoculation with strains Sp245 and Cd on tyrosinase activity in roots (by 1.7 and 3.3 times, respectively) and slightly increased it



Copper content in dried biomass of cv. Saratovskaya 29 seedlings grown in the presence of copper ions and *Azospirillum* strains.

* Values differ significantly from noninoculated control, $p \le 0.05$.

Table 3. Peroxidase activity (U/mg of protein) in cv. Saratovskaya 29 seedlings grown in the presence of copper ions and *Azospirillum* strains

Treatment	DAZ		ABTS		DAP	
	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}
			Roots			
Noninoculated	66.1 ± 6.5	65.5 ± 12.8	21.8 ± 9.5	24.4 ± 9.1	59.0 ± 6.3	55.2 ± 21.3
Sp245	96.3 ± 11.2*	41.4 ± 10.0*#	33.1 ± 8.2*	15.9 ± 4.2#	72.7 ± 10.5*	28.5 ± 11.1*#
Cd	359.0 ± 93.4*	75.4 ± 8.3#	111.4 ± 13.2*	25.7 ± 11.4#	37.3 ± 18.5*	53.9 ± 17.3
Sp7	71.6 ± 6.7	412.1 ± 23.8*#	20.3 ± 6.1	139.9 ± 14.1*#	45.7 ± 11.3*	388.6 ± 43.7*#
			Shoots			
Noninoculated	15.6 ± 5.8	11.4 ± 2.7	4.2 ± 0.6	2.9 ± 0.8#	9.1 ± 2.3	5.9 ± 2.6#
Sp245	17.2 ± 8.1	30.3 ± 12.1*#	4.3 ± 1.1	5.2 ± 2.7*	10.9 ± 3.8	18.6 ± 7.1*#
Cd	15.7 ± 6.5	23.6 ± 7.1*#	4.3 ± 1.7	5.8 ± 1.3*	9.9 ± 2.6	15.8 ± 3.4*#
Sp7	109.9 ± 31.0*	18.5 ± 4.3*#	24.5 ± 6.2*	4.7 ± 1.5*#	68.3 ± 15.0*	14.7 ± 5.1*#

Note. Test substrates: DAZ, *o*-dianisidine; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ammonium; DAP, 2,7-diaminofluorene.

Table 4. The activity of copper-containing phenol oxidases (laccase and tyrosinase) in cv. Saratovskaya 29 seedlings grown in the presence of copper ions and *Azospirillum* strains

Treatment	Laccase				Tyrosinase	
	DAP		SGZ		DOPA	
	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}
			Roots			
Noninoculated	1.7 ± 0.4	2.5 ± 0.8	2.1 ± 0.6	2.4 ± 0.7	4.5 ± 1.7	7.6 ± 2.4#
Sp245	2.6 ± 0.8*	1.4 ± 0.7#	3.2 ± 0.9*	2.4 ± 0.4#	6.9 ± 2.1*	4.0 ± 1.5#
Cd	10.4 ± 2.3*	3.5 ± 1.8*	9.6 ± 1.4*	2.4 ± 0.8#	25.2 ± 3.5*	7.6 ± 1.6#
Sp7	1.7 ± 0.1	11.2 ± 2.4*#	2.3 ± 0.5	16.1 ± 4.2*#	4.0 ± 1.1	28.6 ± 2.2*#
			Shoots			
Noninoculated	0.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.3	1.1 ± 0.1	0.6 ± 0.1#
Sp245	0.5 ± 0.0*	0.5 ± 0.0*	0.7 ± 0.3	0.5 ± 0.0*	1.2 ± 0.1	1.5 ± 0.7*#
Cd	0.5 ± 0.1	0.9 ± 0.2*	0.7 ± 0.2	0.5 ± 0.1	1.1 ± 0.1	1.6 ± 0.2*#
Sp7	2.5 ± 0.6*	2.7 ± 1.0*	5.3 ± 1.3*	2.5 ±0.2*	7.4 ± 1.3*	1.5 ± 0.0*#

Note. Test substrates: DAP, 2,7-diaminofluorene; SGZ, syringaldazine; DOPA, 3,4-dihydroxyphenyl-L-alanine.

in shoots. By contrast, in the presence of copper, strain Sp7 promoted tyrosinase activity in roots (7-fold) and decreased it in shoots (5-fold).

Discussion

Copper is an essential trace element. Excessive copper, however, inhibits plant growth and causes metabolic disorders (Yruela, 2005; Michaud et al., 2007; Wang H. et al., 2011; Pichhode, Nikhil, 2015). This element is widely involved in various physiological processes (photosynthesis, respiration, antioxidant response, hormonal signaling), and a violation of the copper balance can lead to multiple damage to the plant. The mechanism of the toxicity of copper is associated with its ability to bind strongly to oxygen, nitrogen, and sulfur atoms, which, under conditions of excess copper, gives rise to additional bonds and/or substitution of other metals with copper in various biomolecules, including in the active centers of many enzymes (Yruela, 2005; Wang H. et al., 2011). Copper toxicity to plants is manifested as inhibition of growth and signs of chlorosis and is accompanied by oxidative stress. The copper uptake and content in plants depend on several factors, including cultivar differences (Medvedev, Derevyagin, 2017).

In this work, all experiments were conducted with one cultivar of soft spring wheat. This means that the inoculation and copper effects found for cultivar Saratovskaya 29 may not be manifest in other wheat cultivars. Therefore, further studies on different wheat genotypes are needed.

Copper at 0.05 mmol/L affected mainly the wheat seedling roots, which were in direct contact with the toxicant. Wang H. et al. (2011) reported decreases in the length and weight of wheat roots grown hydroponically in the presence of 0.05 mmol/L of copper. Shoot length was also reduced, a trend noted in this study as well. Yet, the copper concentration used had no noticeable effect on the photosynthetic apparatus of wheat. This was concluded from the absence of significant changes in the leaf content of chlorophylls and carotenoids under the effect of the metal (see Table 2). Consequently, photosynthesis was undisturbed by copper toxicity to wheat roots.

The normal physiological concentration of copper in plants ranges from 3 to 30 mg/kg (Wang H. et al., 2011). Wheat is able to take up copper from soil, and roots accumulate larger amounts of copper than does aboveground biomass (Sayyad et al., 2009). Increased metal absorption is an undesirable property of food grains. Liu et al. (2021) examined the genetic mechanisms of metal accumulation by plants by using 246 wheat cultivars and two metals – copper and zinc. They showed that some cultivars are the least prone to the accumulation of toxic elements. The uptake of metals by plants from soil is affected by microbial activity, as well as by numerous organic and inorganic compounds released by roots and present in soil solution (Wang S. et al., 2017). Microbes produce extracellular polymer compounds that can adsorb or chelate metal ions (Yaneva, 2009); as a result, metals are deposited into the medium and are taken up by roots in greater amounts (Wang S. et al., 2017).

Our results show that inoculation of wheat with *A. brasilense* contributed to copper accumulation in plant tissues. The degree of influence of the inoculants (Sp7 > Cd > Sp245) on this variable is probably related to the differences in the root colonization strategy between bacteria. Strain Sp245 is an endophyte, whereas strain Sp7 is an epiphyte (Rothballer et al., 2003). The available information about strain Cd is contradictory: de Oliveira Pinheiro et al. (2002) failed to observe wheat root penetration by this strain, whereas Caiola et al. (2004) observed Cd cells in the tissues of tomato roots.

Here, *A. brasilense* inoculation of wheat contributed to increased plant growth, which was manifested mainly as increased length and weight of roots and shoots. Yet, the

strains differed in their ability to promote plant growth. The endophytic strain Sp245 inhibited root growth in the absence of copper but compensated for the inhibitory effect of copper in its presence. Strain Cd had the greatest effect on the length and weight of the seedlings grown both in the presence and in the absence of copper.

In turn, copper accumulation in wheat tissues was toxic to plants. All inoculated plants showed a sharp twofold decrease in the content of chlorophyll *b* and carotenoids (see Table 2), which could not but disturb the photosynthesis apparatus. It is known that high concentrations of copper can suppress photosynthesis, disrupting the architecture of thylakoid membranes, changing the whole ultrastructure of chloroplasts, and inhibiting the accumulation of chlorophyll and the electron transport of both PS I and PS II (Rai et al., 2016). The toxic effect of copper on the photosynthesis apparatus may be associated with inhibition of the activity of biosynthesis enzymes and with the displacement of Mg^{2+} from the chlorophyll molecule (Prasad M.N.V., Strzalka, 1999; Rai et al., 2016). When the content of the main photosynthetic pigment chlorophyll *a* decreases (which is what was observed when strain Cd was used for inoculation), the auxiliary chlorophyll *b* converts to chlorophyll *a*. As a result, the concentration of chlorophyll *b* decreases to a greater extent than does the concentration of chlorophyll *a*, and ultimately, the chlorophyll *a*/*b* ratio increases (Breckle, 1991; Prasad D.D.K., Prasad A.R.K., 1987). With strains Sp245 and Sp7, which did not affect the chlorophyll *a* content in the inoculated leaves in the presence of copper, the chlorophyll *a*/*b* ratio may have increased owing to the photochemical oxidation of the light-harvesting complexes binding chlorophyll *b* (Huang et al., 2004).

As a rule, environmental stress caused by both biotic and abiotic factors leads to the formation and accumulation of reactive oxygen species in plant cells, which damage the cells and interfere with plant growth and yields. It is known that heavy metals can induce plant oxidative stress, the mechanisms and responses to which have been repeatedly described (Titov et al., 2014). In addition, the ability of bacteria to cause oxidative stress has been well documented (Rais et al., 2017). In response to oxidative explosion, various forms of plant antioxidants are activated, among which an important part is played by antioxidant defense enzymes. The activation of these enzymes under heavy metal stress has been described in detail (Titov et al., 2014). Rais et al. (2017) proposed that under stress caused by microbial infection, the antioxidant enzymes are activated in response to the recognition of microbial molecular patterns by the plant immune system; to some secondary metabolites of the microorganisms; and to plant iron status, altered by microbial siderophores. The changes in the activity of the antioxidant enzymes in wheat in response to various stress factors were summarized by Caverzan et al. (2016).

This study has shown how *Azospirillum* strains that use different strategies to colonize wheat roots in the presence of copper can affect the activity of peroxidase – an enzyme that, owing to its specific properties and a great variety its molecular forms, is a key protective cellular system that is used when any stress factors affect the plant (Statsenko et al., 2008). We have found that the activity of the peroxidase of wheat seedlings was significantly higher in roots than in shoots. The copper concentration used did not affect the activity of peroxidase in the roots and only slightly reduced it in the shoots of non-inoculated plants. In turn, inoculation significantly changed peroxidase activity both in the absence and in the presence of copper. The inoculation effect was strain-specific. In plants grown without copper, peroxidase activity was significantly increased in roots when strains Sp245 and Cd were used and in shoots when strain Sp7 was used. By contrast, with copper, strain Sp7 strongly induced peroxidase activity in roots and to a lesser extent in shoots, whereas strains Sp245 and Cd promoted peroxidase activity mainly in shoots. Thus, inoculation caused a pronounced antioxidant stress response, in which various peroxidase isoforms were probably involved. The increase in copper uptake by the inoculated plants additionally promoted peroxidase activity, which shows a potentiated effect of the abiotic and biotic stress factors.

Besides peroxidases, phenol oxidases are almost universally present in plants. They are often induced under stress caused by damage to plants or by pathogen attack and are important for plant defense response (Sullivan, 2015). There is evidence (Yang et al., 2007) that biotic and abiotic stress activates plant lignin synthesis, which involves phenol oxidase and peroxidase. Our data show that the activity of these enzymes was affected by inoculation to a greater extent than it was affected by copper. Inoculation promoted an increase in the tissue concentration of copper, which, as a rule, promoted the activity of the phenol oxidases both as copper-dependent enzymes and as stress enzymes.

The search for correlations between plant treatments and the variables analyzed showed a significant close correlation between the change in enzyme activity and the *Azospirillum* inoculation of plants ($r_s = 0.76$, $p < 0.05$). It is noteworthy that among the strains tested, only the epiphyte *A. brasilense* Sp7 increased root enzyme activity in the presence of copper.

Thus, all the strains tested contributed to the uptake of copper by plants. We emphasize that the differences in the effects observed were caused by different strains. Thus, without copper, the endophytic strain *A. baldaniorum* Sp245 reduced the length but increased the weight of the seedling roots, contributed the least to the uptake of copper, and caused the least induction of the antioxidant enzymes and phenol oxidases. Inoculation of plants with the epiphytic strain *A. brasilense* Sp7 in the absence of copper increased shoot peroxidase and oxidase activity the most and contributed the most to the uptake of copper and to the activation of root peroxidases and oxidases in the presence of copper. The in-between strain *A. brasilense* Cd promoted wheat growth the most, regardless of the presence of copper. Without copper, this strain increased root peroxidase and oxidase activity the most, and in the presence of copper, it inhibited the plant photosynthesis apparatus the most.

Conclusion

Overall, the obtained results clearly show that the effect of *Azospirillum* on the physiological and biochemical status of wheat is diverse. The compensatory effect of bacteria on copper toxicity and the simultaneous increase in metal accumulation in plant tissues can be considered as mutually exclusive crop-production aspects associated with the growing of food plants in heavy-metal-polluted areas.

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Virulence potential of faecal *Escherichia coli* strains isolated from healthy cows and calves on farms in Perm Krai

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Abstract. Cattle are a reservoir of pathogenic and potentially pathogenic *Escherichia coli* (*E. coli*) strains, which can pose a threat to human and animal health. The aim of the study was to evaluate the occurrence of 22 virulence-associated genes (VAGs), as well as the prevalence of antimicrobial drug resistance and three different *bla*-genes among 49 *E. coli* strains isolated from healthy cattle. The presence of VAGs that are common among diarrheagenic *E. coli* (DEC) strains and/or extraintestinal pathogenic *E. coli* (ExPEC) strains was determined by amplifying specific gene sequences by PCR. The following VAGs associated with DEC were found: *east1* in 24.5 % of the studied *E. coli* strains, *estI* in 10.2 %, *ehxA* in 8.2 %, *stx2* in 6.1 %, *eltA* in 4.1 %, *estII* and *stx1* in 2.0 % of the studied strains. The prevalence of ExPEC VAGs was: *fimH* – 91.8 %, *afa/draBC* – 61.2 %, *iutA* – 44.9 %, *flu* – 32.7 %, *sfaDE* and *hlyF* – 30.6 %, i*roN* – 22.4 %, *ompT* and *papC* – 20.4 %, *kpsMTII* and *hlyA* – 18.4 %, *iss* – 14.3 %, *usp* – 2.0 %, *cnf1* and *iha* were not detected among the studied strains. Based on the found co-occurrence of VAGs "classical", hetero-pathogenic and hybrid-pathogenic *E. coli* strains were found. *E. coli* strains isolated from cows had a higher diarrheagenic potential, whereas *E. coli* strains isolated from calves more frequently contained genes associated with the ExPEC pathotype. Among the studied *E. coli* strains, 77.6 % were resistant to ampicillin, 49.0 % to tetracycline, 20.4 % to chloramphenicol, 16.3 % to cefoperazone, 16.3 % to ceftriaxone, 16.3 % to aztreonam, 14.3 % to cefepime, 10.2 % to norfloxacin, 10.2 % to ciprofloxacin, 6.1 % to levofloxacin and 2.0 % to gentamicin. All strains were sensitive to meropenem and amikacin. 32.7 % of the studied *E. coli* strains were found to be multidrug resistant, as they were resistant to at least three groups of antibiotics. With PCR, the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes were detected in 100, 31.6, and 26.3 %, respectively, of strains resistant to at least one of the beta-lactam antibiotics. Thus, it was shown that the studied faecal *E. coli* of healthy cows and calves had a high hetero-pathogenic potential, therefore in the future molecular genetic characterization of these bacteria shall be an important part of the epizootic monitoring.
Key words: *Escherichia coli*; virulence-associated genes (VAGs); antibiotic resistance; cattle.

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Патогенный потенциал интестинальных штаммов *Escherichia coli*, выделенных от здоровых коров и телят в хозяйствах Пермского края

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Аннотация. Крупный рогатый скот является резервуаром патогенных и потенциально патогенных *Escherichia coli* (*E. coli*), которые могут представлять угрозу для здоровья людей и животных. Цель исследования – оценить встречаемость 22 вирулент-ассоциированных генов, а также распространенность антибиотикоустойчивости и трех генов *bla* различных типов среди штаммов *E. coli*, выделенных от здорового крупного рогатого скота. Сорок девять штаммов *E. coli* были проанализированы методом ПЦР на присутствие генов, распространенных среди представителей диареегенной *E. coli* (DEC) и внекишечной патогенной *E. coli* (ExPEC). Обнаружены следующие детерминанты, ассоциированные с DEC: *east1* – 24.5 %, *estI* – 10.2 %, *ehxA* – 8.2 %, *stx2* – 6.1 %, *eltA* – 4.1 %, *estII* и *stx1* – 2.0 %. Распространенность генов ExPEC составила: *fimH* – 91.8 %, *afa/draBC* – 61.2 %, *iutA* – 44.9 %, *flu* – 32.7 %, *sfaDE* и *hlyF* – 30.6 %, *iroN* – 22.4 %, *ompT* и *papC* – 20.4 %, *kpsMTII* и *hlyA* – 18.4 %, *iss* – 14.3 %, *usp* – 2.0 %, *cnf1* и *iha*

не детектированы. На основании установленных комбинаций генов были определены «классические», гетеропатогенные и гибридные штаммы. Эшерихии, выделенные от коров, обладали более высоким диареегенным потенциалом, а *E. coli*, изолированные от телят, чаще содержали гены, ассоциированные с патотипом ExPEC. Среди исследованных штаммов были устойчивыми: к ампициллину – 77.6 % культур, тетрациклину – 49.0 %, хлорамфениколу – 20.4 %, цефоперазону, цефтриаксону, азтреонаму – 16.3 %, цефепиму – 14.3 %, норфлоксацину и ципрофлоксацину – 10.2 %, левофлоксацину – 6.1 %, гентамицину – 2.0 %, все штаммы были чувствительны к меропенему и амикацину. Фенотип множественной лекарственной устойчивости имели 32.7 % культур, так как они были устойчивы к трем и более группам антибиотиков. Специфическая амплификация выявлена: к bla_{TEM} – у 100 % штаммов, к bla_{SHV} – у 31.6 %, к bla_{CTX-M} – у 26.3 % штаммов, устойчивых хотя бы к одному препарату из группы бета-лактамных антибиотиков. Штаммы *E. coli*, изолированные от здоровых коров и телят, обладали высоким гетеропатогенным потенциалом, поэтому молекулярно-генетическая характеристика этих бактерий должна стать важной частью эпизоотического мониторинга.
Ключевые слова: *Escherichia coli*; гены, ассоциированные с вирулентностью; устойчивость к антибиотикам; крупный рогатый скот.

Introduction

Representatives of the commensal microbiota, including *Escherichia coli*, being obligate residents of the intestinal tract of farm animals, support physiological homeostasis and colonization resistance of the organism. At the same time, cattle, including healthy animals, present a reservoir of pathogenic and opportunistic *E. coli* (Chapman et al., 2006; Ewers et al., 2009; Bok et al., 2015; Madoshi et al., 2016). Diarrheagenic *E. coli* (DEC) causing outbreaks of intestinal diseases include various pathotypes: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC), which include also Shiga toxin-producing *E. coli* (STEC) (Allocati et al., 2013; Vila et al., 2016; Oporto et al., 2019; Santos et al., 2020).

Extraintestinal pathogenic *E. coli* (ExPEC) are usually divided according the infected organ system, e. g. uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SePEC). Intestinal and extraintestinal *E. coli* strains circulating in agricultural enterprises can pose a significant health risk to animals and humans.

Due to horizontal gene transfer, the *E. coli* genome is highly heterogeneous, and strains possessing genes characteristic of different pathotypes, so called hybrid-pathogenic and hetero-pathogenic *E. coli*, are known (Santos et al., 2020). Along with this, the pathogenic potential of intestinal *E. coli* is formed, which become sources of virulence-associated genes (VAGs) for other microorganisms, or, subsequently, themselves cause intestinal or extraintestinal infections (Chapman et al., 2006; Bélanger et al., 2011).

The widespread use of antibiotics in agriculture leads to the formation of *E. coli* strains with a multidrug resistance (MDR) phenotype (Pardon et al., 2017). The relationship between pathogenicity determinants and antimicrobial resistance controversial: in a number of studies, a correlation between phenotypic antibiotic resistance and the presence of certain VAGs was revealed (Suojala et al., 2010; de Verdier et al., 2012), in other studies this relationship was absent (Bok et al., 2015). In Russia, studies on the occurrence of hybrid-pathogenic and hetero-pathogenic strains of *E. coli* circulating among healthy animals of agricultural enterprises have not been conducted. In this regard, the analysis of the genetic profiles of pathogenicity and antibiotic resistance of *E. coli* strains, obligate representatives of the intestinal microbiota of cattle, is important in relation to both epizootic and epidemiological control of colibacillosis in livestock farms.

The aim of the study was to evaluate the occurrence of 22 VAGs, as well as the prevalence of antibiotic resistance and three different types of *bla*-genes among *E. coli* strains isolated from faeces of healthy cattle.

Materials and methods

Studied strains. In the study, 49 different strains of *E. coli* (non-clonality of the strains was ascertained by ERIC-PCR), isolated in 2019–2021 at agricultural enterprises (n = 3) and private farms (n = 5) in Perm Krai from the faeces of cows (n = 31) and calves from 3 to 13 days of age (n = 18) were included. The strains were obtained from different animals of the Holstein black-and-white breed. The agricultural enterprises LLC "Krasava", LLC "Serginskoe" and LLC "Rus" specialize in dairy cattle breeding and raw milk production. The economic diet of feeding and the conditions of keeping animals (loose method) are the same and typical for these enterprises.

Detection of virulence-associated genes. To obtain matrix DNA for PCR amplification, a loop of bacterial biomass was resuspended into 100 µL of ultrapure water, heated for 15 min at 97 °C in a solid-state thermostat with a timer TT-2 "Termite" (Russia), centrifuged for 5 min at 13,000 rpm. The supernatants were transferred to fresh Eppendorf tubes and stored at –20 °C until usage. Twenty-two genes encoding either toxins (*hlyA*, *hlyF*, *east1*, *ehxA*, *estI*, *estII*, *eltA*, *stx1*, *stx2*, *cnf1*), adhesins (*fimH*, *papC*, *sfaDE*, *afa/draBC*, *iha*, *flu*), protectins (*ompT*, *kpsMTII*, *iss*), proteins of iron uptake systems (*iroN*, *iutA*) or the UPEC-specific protein (*usp*) were detected by PCR. Primers (LLC "Sintol", Russia) and programs according to the recommendations of the authors (Chapman et al., 2006; Moulin-Schouleur et al., 2007) were used. Amplifications were carried out in PCR mixtures with Taq-polymerase (LLC "Sintol") in a thermal cycler DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). Band visualization and data documentation were performed using a gel documentation system Gel-DocXR (Bio-Rad).

Antimicrobial susceptibility testing. The determination of the sensitivity of *E. coli* strains to antibiotics was carried out in accordance with the methodical instructions MUK 4.2.1890-04 (Russia, 2004) and the clinical guidelines "Determination of the Sensitivity of Microorganisms to Antimicrobial Drugs" of

the Interregional Association for Clinical Microbiology and Antimicrobial Chemotherapy (IACMAC, version-2018-03). The strains were tested by the disk-diffusion method using Muller–Hinton agar (FBIS SRCAMB, Russia) and disks (NICF, St. Petersburg, Russia) for sensitivity to penicillins (ampicillin, 10 μg), cephalosporins (cefoperazone, 75 μg; ceftriaxone, 30 μg; cefepime, 30 μg), carbapenems (meropenem, 10 μg), monobactams (aztreonam, 30 μg), aminoglycosides (amikacin, 30 μg; gentamicin 10 μg), fluoroquinolones (ciprofloxacin, 5 μg; levofloxacin, 5 μg; norfloxacin, 10 μg), tetracyclines (tetracycline, 30 μg), phenicols (chloramphenicol, 30 μg). Resistance of *E. coli* strains to at least one drug of three or more groups of antibiotics was defined as multidrug resistance (Magiorakos et al., 2012).

Identification of beta-lactamase genes. Detection of genes encoding TEM, SHV, and CTX-M beta-lactamase types was carried out with PCR using primers and amplification modes, according to the recommendations of the authors (Ahmed et al., 2007; Aleisa et al., 2013) with the same PCR mixtures and machines as stated above for detection of virulence-associated genes.

Statistical analysis. Qualitative features were compared using χ^2 (with Yates correction) or Fisher's exact test. Data processing was carried out using computer programs Microsoft Office XP Excel and Statistica 10.0.

Results

Molecular characteristics of the *E. coli* strains

Evaluation of the prevalence of genes associated with DEC (*east1*, *ehxA*, *estI*, *estII*, *eltA*, *stx1*, *stx2*) and ExPEC (*fimH*, *papC*, *sfaDE*, *afa/draBC*, *flu*, *hlyA*, *hlyF*, *ompT*, *kpsMTII*, *iss*, *iroN*, *iutA*, *usp*) showed that they occurred with different frequencies. The *iha* and *cnf1* genes were not detected (Table 1). All strains contained at least one VAG. The most *E. coli* were harbouring three (20.4 %), four (14.3 %), five (20.4 %) and six (16.3 %) genes, while the proportion of *E. coli* having seven or more genes did not exceed 10 %. In total, forty-five variants of VAGs combinations were identified.

Prevalence of genes associated with DEC pathogenicity. Seventeen strains (34.7 %) contained genes associated with DEC pathotypes. Among the toxin-coding genes, the most common was the enteroaggregative thermostable enterotoxin *east1* gene (24.5 %), which is usually, but not exclusively, associated with EAEC. Seven strains (14.3 %) carried genes associated with ETEC (*estI*, *estII*, *eltA*), four cultures contained STEC-marker genes *stx1* (2.0 %) and *stx2* (6.1 %). In four cases, *ehxA* was found, encoding enterohemolysin, which is the main virulence factor of EHEC, but also occurs among other diarrheal *E. coli* pathotypes (Jiang et al., 2015). A hetero-pathogenic strain that simultaneously contains marker genes for STEC and ETEC pathotypes was found. It should be noted that the *east1* gene was detected in some *E. coli* strains identified as STEC and ETEC. The distribution of determinants associated with DEC pathotypes in the studied *E. coli* population is shown in Fig. 1.

Prevalence of genes associated with ExPEC pathogenicity. The *fimH* gene was the most abundant (91.8 %). The second most common gene was the afimbrial adhesin *afa/draBC* (61.2 %); also quite often *iutA* was detected (44.9 %). The prevalence of the *papC*, *sfaDE*, *flu*, *hlyA*, *hlyF*, *ompT*, *kpsMTII*, *iss*, *iroN* genes varied from 14.3 to 32.7 %. Only in one case the *usp* gene was detected.

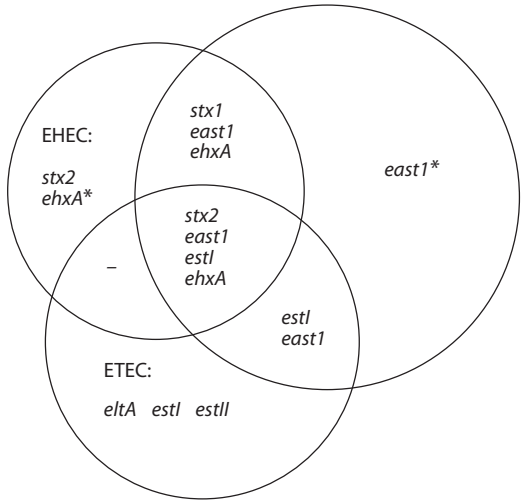

Fig. 1. Variants of the distribution of genes associated with DEC pathotypes.

* The gene is associated with more than one pathotype.

More than half of the strains (55.1 %) corresponded to the ExPEC group according to the classification criteria of J.R. Johnson and T.A. Russo (2005); that is, they contained two or more of the following genes: *papC*, *sfaDE*, *afa/draBC*, *kpsMII*, *iutA*. Interestingly, eight strains included at least three of the five genes (*hlyF*, *iroN*, *ompT*, *iss*, *iutA*) that were proposed by T.J. Johnson et al. (2008) to determine the APEC pathotype associated with systemic avian colibacillosis. One strain had a high uropathogenic potential because it contained the *usp* gene, as well as the *hlyA*, *papC*, *sfaDE*, *afa/draBC* genes often found among UPEC strains.

Based on the detected combinations of genes, not only "classic" but also hybrid-pathogenic strains were identified. Eleven (22.5 %) cultures were identified that met the ExPEC criterion and included genes associated with DEC pathotypes (*estI*, *stx2*, *east1*, *ehxA*). Among them, hybrid pathotypes ExPEC/STEC and ExPEC/ETEC were found, but the prevalence of such strains did not exceed 4.1 %. The ratio of genes associated with ExPEC and DEC detected in the studied *E. coli* population is shown in Fig. 2.

Comparison of the prevalence of VAGs in subpopulations of *E. coli* isolated from cows and calves. Some statistical differences in the prevalence of VAGs between *E. coli* from samples of cows and calves were found (see Table 1). The *iss* gene was detected only among *E. coli* isolated from calves, while the *stx1*, *stx2*, *ehxA*, *estII*, *hlyA* and *usp* genes were found exclusively in *E. coli* isolated from cows. The *ompT* gene was found significantly more often in *E. coli* circulating among calves ($p = 0.03$), while the prevalence of the *afa/draBC* ($p = 0.03$) and *iroN* ($p = 0.04$) genes was higher in subpopulations of *E. coli* isolated from cows. In addition, the *fimH*, *papC*, *sfaDE*, *estI*, *east1*, *kpsMTII* genes were more common among the latter, but the difference was not statistically significant (Fig. 3).

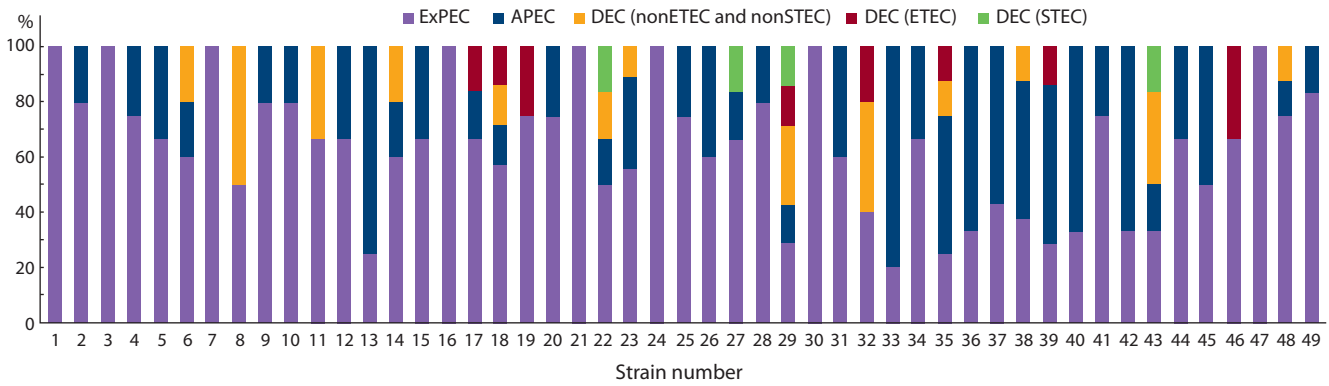

Fig. 2. The ratio of genes associated with APEC, STEC, ETEC pathotypes, other ExPEC and DEC genes in strains isolated from healthy cows and calves.

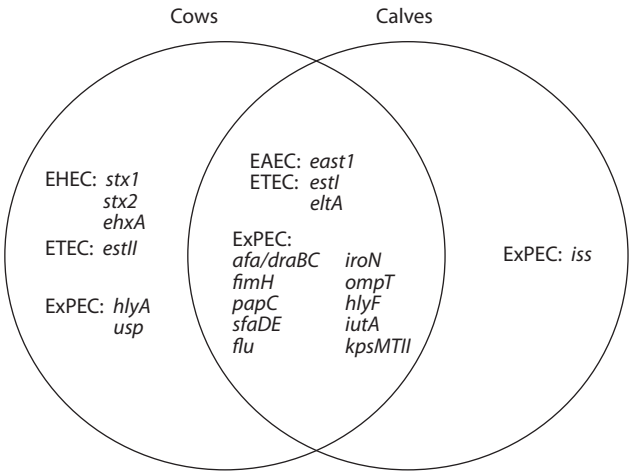

Fig. 3. Distribution of pathogenicity determinants among strains isolated from healthy cows and calves.

Characterization of antimicrobial resistance of *E. coli* strains

The proportion of strains sensitive to all studied antibiotics was 12.2 %. *E. coli* strains resistant to only one drug were the most common in the population (36.7 %). Cultures were more often resistant to ampicillin (77.6 %) and tetracycline (49.0 %) (Table 2). It should be noted that all strains were sensitive to meropenem and amikacin.

Sixteen strains (32.7 %) had an MDR phenotype, while three strains were resistant to at least one antimicrobial agent from five or more groups of antibiotics. Of the fourteen identified phenotypic profiles of antibiotic resistance, seven were unique (not repeated more than once). The most common were strains with the phenotype of resistance to ampicillin (32.7 %), ampicillin and tetracycline (12.3 %), as well as ampicillin, tetracycline and chloramphenicol (10.2 %).

Prevalence of beta-lactam resistance genes. Thirty-eight *E. coli* strains (77.6 %) were resistant to at least one beta-lactam antibiotic. These strains were tested for the presence of beta-lactamase genes. Specific amplification for bla_{TEM} was detected in 100 % of cases, for bla_{SHV} – 31.6 %, for bla_{CTX-M} – 26.3 %.

Comparative analysis of the prevalence of drug resistance in subpopulations of *E. coli* isolated from cows and calves. It should be noted that strains resistant to gentamicin and norfloxacin were found only among *E. coli* obtained from calves. In the same group, the occurrence of *E. coli* representatives that were not sensitive to tetracycline and chloramphenicol, as well as those with the MDR phenotype, was significantly higher ($p < 0.01$). The proportion of strains resistant to other antimicrobial agents was also higher in the calf group, although the differences were not statistically significant (see Table 2).

Relationship between virulence factors and antimicrobial resistance

In the group of strains with the MDR phenotype, *E. coli* containing five or more VAGs were found more often ($p = 0.04$), and the probability of finding the *hlyA*, *iss*, *iutA* genes in this group was higher than among *E. coli* without the MDR phenotype ($p \leq 0.05$). In the group of strains in which five or more pathogenicity genes were detected, the proportion of *E. coli* resistant to five or more antimicrobial agents was significantly higher ($p = 0.04$). It should be noted that among *E. coli* with the MDR phenotype, there were *E. coli* containing the marker genes *estI*, *eltA* (ETEC), *stx1* (STEC), as well as six strains identified as APEC.

Discussion

E. coli strains circulating in agricultural settings can pose a significant risk to human health (Bélanger et al., 2011; Manges et al., 2016). On the one hand, the possibility of transmission of pathogenic *E. coli* through food products, including cattle meat, has been revealed (Vincent et al., 2010). On the other hand, the presence of similar phylogroups, serotypes and genetic determinants of pathogenicity in representatives of *E. coli* that cause human diseases and *E. coli* of animal origin suggests that animals can be a reservoir of opportunistic *E. coli*, as well as pathogens of zoonotic infections (Tivendale et al., 2010; Mora et al., 2013). For example, farm animals are the main natural reservoir and source of STEC strains that cause hemorrhagic colitis in humans (Onishchenko et al., 2015).

Table 1. Occurrence of virulence-associated genes among *E. coli* strains isolated from faeces of healthy animals

Virulence factor	Gene	Pathotype	Frequency, %		
			Cows	Calves	All animals
Adhesion factors					
Bifunctional enterobactin receptor adhesin	*iha*	EHEC	0	0	0
Antigen Ag43a	*flu*	ExPEC	25.8	44.4	32.7
Afimbrial adhesin	*afa/draBC*	ExPEC	74.2	39.8*	61.2
Type 1 fimbriae	*fimH*	ExPEC	96.8	83.3	91.8
P-fimbriae	*papC*	ExPEC	22.6	16.7	20.4
S-fimbriae	*sfaDE*	ExPEC	32.3	27.8	30.6
Toxins					
Hemolysin A	*hlyA*	ExPEC	29.0	0	18.4
Hemolysin F	*hlyF*	ExPEC	19.4	30.0	30.6
Heat-labile enterotoxin	*eltA*	ETEC	3.2	3.6	4.1
Heat-labile enterotoxin a	*est1*	ETEC	12.9	5.6	10.2
Heat-labile enterotoxin b	*estII*	ETEC	3.2	0	2.0
Cytotoxic necrotizing factor	*cnf1*	ExPEC	0	0	0
Shiga-like toxin type 1	*stx1*	STEC	3.2	0	2.0
Shiga-like toxin type 2	*stx2*	STEC	9.7	0	6.1
Enteroaggregative heat-stable enterotoxin	*east1*	EAEC	22.6	27.8	24.5
Enterohemolysin	*ehxA*	EPEC, EHEC	12.9	0	8.2
Iron uptake					
Salmochelin siderophore	*iroN*	ExPEC	32.3	5.6	22.4
Aerobactin siderophore receptor	*iutA*	ExPEC	25.8	77.8	44.9
Protectins					
Increased serum survival protein	*iss*	ExPEC	0	38.9	14.3
Group II capsular antigen	*kpsMTII*	ExPEC	22.6	11.1	18.4
Outer membrane protease	*ompT*	ExPEC	9.7	39.8*	20.4
Other factors					
Uropathogenic-specific protein	*usp*	ExPEC	3.2	0	2.0

* The difference between the samples was statistically significant, $p \leq 0.05$.

The presence of certain virulence factors in the pathogen causes the manifestation of clinical symptoms of intestinal and extraintestinal infections caused by *E. coli*, the corresponding pathological groups – DEC and ExPEC (Chapman et al., 2006; Dale, Woodford, 2015). According to numerous studies, these strains can circulate among the microbiota of healthy animals that do not have pronounced symptoms of the disease, in addition, some VAGs may be present in the genomes of commensal *E. coli* (Orden et al., 2002; Ewers et al., 2009, 2021; Bok et al., 2015). Our studies showed that *E. coli* strains isolated from healthy cattle were characterized by a high level of genetic diversity and contained pathogenicity determinants associated with pathotypes DEC and ExPEC. ExPEC strains were the most common, as they were found in 55.1 % of the studied strains. *E. coli* containing marker genes of diarrheagenic pathotypes: STEC (in 8.1 % of cases) and ETEC (14.3 %) were also detected. Similar data were presented in the study by J.A. Orden et al. – among the strains obtained from healthy cattle, there were representatives of STEC and EPEC with frequencies of 8.7 and 8.2 %, respectively (Orden et al., 2002), whereas the prevalence of ETEC and STEC representatives isolated from dairy cows in China was only 4.29 and 1.98 % (Huasai et al., 2012). It should be noted that in our sample, individual VAGs were detected with a high frequency (*fimH* – 91.8 %; *afa/draBC* – 61.2 %; *iutA* – 44.9 %; *sfaDE* – 30.6 %). R.V. Pereira et al. (2011) found that the *fimH* and *iutA* genes were more prevalent among *E. coli* isolated from healthy calves – in 100 and 86.9 % of cases respectively, while the *sfaDE* and *afa/draBC* genes were found less frequently – in 4.9 and 1.6 % of cases, respectively.

When comparing the prevalence of pathogenicity determinants in strains circulating among healthy cattle of Russian and Slovenian farms, it was found that faecal *E. coli* strains from Slovenian cows had a lower virulence potential, since the occurrence of VAGs was significantly lower: *fimH* – 65.2 %,

Table 2. Prevalence of antibiotic resistance

Groups of antibiotics	Antimicrobial agent	Resistant strains, %		
		Cows	Calves	All animals
Penicillins	Ampicillin	71.0	88.9	77.6
Cephalosporins	Cefoperazone	12.9	22.2	16.3
	Ceftriaxone	12.9	22.2	16.3
	Cefepime	9.7	22.2	14.3
Monobactams	Aztreonam	12.9	22.2	16.3
Carbapenems	Meropenem	0	0	0
Aminoglycosides	Gentamicin	0	5.6	2.0
	Amikacin	0	0	0
Fluoroquinolones	Levofloxacin	3.2	11.1	6.1
	Norfloxacin	0	27.8	10.2
	Ciprofloxacin	3.2	22.2	10.2
Tetracyclines	Tetracycline	22.6	94.4*	49.0
Phenicols	Chloramphenicol	3.2	50*	20.4

* The difference between the samples was statistically significant, $p \leq 0.05$.

hlyA – 9.0 %, *stx2*, *ompT* and *kpsMT* – 3.4 %, *usp* – 1.1 %, and the *sfaDE*, *iroN*, *cnf1* genes were not detected at all (data not shown).

Recently, more researchers have noted that VAGs associated with either ExPEC or DEC are found among atypical *E. coli* pathotypes (Santos et al., 2020; Ewers et al., 2021). Such strains can cause severe infectious diseases in both farm animals and humans. In 2011, an outbreak of food poisoning was recorded in Germany, caused by a hetero-pathogenic strain of *E. coli* O104 : H4 with a rare combination of VAGs (*stx2* and *aatA*, *aggR*, *aar*, *aggA*, *aggC*), characteristic of two different groups of diarrheagenic *E. coli* – STEC and EAEC (Bielaszewska et al., 2011). It was reported that hetero-pathogenic strains can be isolated from animals and food (Cheng et al., 2006; Monday et al., 2006).

In our study, strains were found that included the *stx1*, *stx2* genes and the gene of enteroaggregative thermostable enterotoxin *east1*, which is often found in EAEC strains. However, to determine this pathotype, it is necessary to identify additional determinants, and also to perform phenotypic studies (Boisen et al., 2020). ExPEC/STEC hybrids are also high-risk pathogens because they cause both diarrhoea and extraintestinal infection. We found hybrid-pathogenic and hetero-pathogenic strains in 2.0 and 4.1 % of cases, respectively.

Our study revealed that the VAG profiles of *E. coli* strains circulating among healthy cows and calves had specific differences. The occurrence of VAGs (except for *ompT*, *hlyF*, *iutA*) was higher among *E. coli* isolated from cows; moreover, genes *stx1*, *stx2*, *ehxA* and *estII* associated with DEC were detected exclusively in this sample. Interestingly, among *E. coli* isolated from calves, the genes *ompT*, *hlyF*, *iutA*, *iss* were detected more often. Thus, *E. coli* living in the intestines of healthy cows had a high diarrheagenic potential, while ExPEC genes were common in both samples; however, in the group of calves, *E. coli* containing genes associated with the APEC pathotype were more common. Perhaps these differences are related to the fact that bacteria of the DEC pathogroup can persist in the intestines of cows without causing active infection, since the "mature" microbiome provides colonization resistance, while calves are more vulnerable to DEC, which often cause diarrhoea and death of young animals in the first days of life (Bashahun, Amina, 2017). In addition, natural immunity formed in previously ill adult animals, as well as post-vaccination immunity, provide tolerance to most pathogenic *E. coli*.

Agriculture accounts for up to 70 % of antimicrobial drug consumption, so productive animals are the main arena for the emergence of bacterial antibiotic resistance and the emergence of strains with multiple drug resistance (Berge et al., 2009; Pereira et al., 2011; Okello et al., 2021). It was shown that among *E. coli* isolates circulating in poultry and agricultural enterprises, more than half had the MDR phenotype[1].

Significant differences in the prevalence of antibiotic-resistant microorganisms circulating in livestock farms in different countries may be due to the peculiarities of animal housing conditions and the use of antimicrobial drugs. This determines the expediency of a comparative study of transmission routes and mechanisms of acquiring antibiotic resistance.

Beta-lactam antibiotics and tetracycline preparations are most widely used in veterinary medicine for treatment and prevention of infectious diseases of cattle (Berge et al., 2009; Pereira et al., 2011). Of particular importance is the growing resistance of microorganisms to extended-spectrum cephalosporins (third and fourth generation), as these antibiotics are

[1] Zabrovskaya A.V. Epizootological analysis of the spread of antibiotic-resistant strains of pathogens of infectious diseases of farm animals in the North-Western federal district of the Russian Federation: Doctor Sci. (Vet.) Dissertation. St. Petersburg, 2019. 323 p.

critically important for medicine[2]. According to our study, strains with the MDR phenotype isolated from healthy cows and calves were found with a high frequency (32.7 %). In the study sample, 77.6 % of cultures were resistant to at least one antimicrobial agent of the beta-lactam group of antibiotics (16.3 % – to cefoperazone and ceftriaxone), 49.0 % – to tetracycline, and 20.4 % – to chloramphenicol. These data significantly exceed the values published by B.P. Madoshi et al. (2016), who found the proportion of strains isolated from healthy cattle and resistant to ampicillin, tetracycline and chloramphenicol was 21.3, 33.1 and 4.4 %, respectively. Only 3.7 % of the strains were resistant to cefotaxime (Madoshi et al., 2016). Even lower resistance to cephalosporins (1.5 %) was demonstrated by *E. coli* strains isolated from cattle faeces at agricultural enterprises in Japan (Sato et al., 2014).

Beta-lactamase production is one of the main mechanisms of resistance to beta-lactam antibiotics. In the studied strains resistant to at least one agent from this group of antimicrobial drugs, genes and combinations of beta-lactamase genes of the TEM, SHV and CTX-M families were found. This fact may be related to the widespread use of beta-lactam antibiotics in enterprises of Perm Krai. However, it was found that even among strains isolated from cattle on farms where antibiotics were rarely used, the occurrence of $bla_{\text{CTX-M}}$ ranged from 2.3 to 25.0 % (Lee et al., 2020). Attention should be paid to the high occurrence in *E. coli* strains of genes encoding beta-lactamases, the plasmid localization of which can contribute to the effective spread of antibiotic resistance within the microbial population through horizontal transfer.

According to our data, in general, resistance to antimicrobial agents was more common in the *E. coli* subpopulation isolated from calves than among *E. coli* isolated from adult animals. The largest differences were observed for tetracycline (94.4 versus 22.6 %) and chloramphenicol (50.0 versus 3.2 % resistant strains from calves and cows, respectively). Perhaps this is due to the addition of these drugs to the calves' feed for a long period, since it is known that antibiotics are often added to milk or milk substitutes in order to prevent diseases and treat diarrhoea, which is the main cause of mortality of calves before weaning (Berge et al., 2009; de Campos et al., 2021; Okello et al., 2021).

It is known that the phenotype of resistance of bacteria circulating among calves is mainly a consequence of the use of antibiotics in enterprises (DeFrancesco et al., 2004; Sato et al., 2005). Antibiotics of the aminoglycoside group – neomycin and gentamicin, are of great importance for the prevention and treatment of streptococcal and staphylococcal infections in calves[3]. This may explain that *E. coli* strains resistant to gentamicin and norfloxacin were found only among *E. coli* derived from calves.

Conclusion

Microbiological monitoring of pathogenic and conditionally pathogenic microorganisms isolated from farm animals and from animal products is currently carried out at all enterprises of the Russian Federation. This monitoring is important, as bacteria in the herd can circulate between animals of all ages over a long period of time, posing a risk to the animals themselves and to the personnel.

This paper presents for the first data on the prevalence of VAGs, as well as the occurrence of hybrid-pathogenic and hetero-pathogenic strains of *E. coli* circulating among healthy animals at agricultural enterprises in the European part of Russia (Perm Krai). In addition, the relationship between the virulence potential of *E. coli* and their antibiotic resistance was analysed. Another important aspect presented in the work is a comparative analysis of the biological properties of *E. coli* strains isolated from different age groups of animals – cows and calves.

Studies have shown that *E. coli* strains circulating among healthy animals on farms and agricultural enterprises were characterized by a high hetero-pathogenic potential. In the *E. coli* population under consideration, representatives of DEC (including STEC and ETEC), which can cause intestinal infections, as well as ExPEC, causing extraintestinal infections, were common. In addition, hybrid strains combining genes associated with different *E. coli* pathotypes were found. Strains with the MDR phenotype had a high virulence potential, since they more often contained more than five VAGs. *E. coli* isolated from cows showed a higher diarrheagenic potential, while *E. coli* isolated from calves more often contained genes associated with the ExPEC pathotype. *E. coli* obtained from calves generally showed greater resistance to antimicrobial agents than *E. coli* isolated from adult animals.

The obtained data on the molecular properties of microorganisms of the intestinal microbiota of healthy cattle allow to assess their epizootic significance and can serve as a basis for the formation of a monitoring system for colibacillosis in agricultural enterprises.

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Effects of antimicrobials on *Pseudomonas aeruginosa* biofilm formation

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Abstract. *Pseudomonas aeruginosa* is one of the most problematic pathogens in medical institutions, which may be due to the ability of this microorganism to exist in a biofilm, which increases its resistance to antimicrobials, as well as its prevalence and survival ability in the external environment. This work aimed to evaluate the antimicrobial susceptibility of *P. aeruginosa* strains in planktonic and biofilm forms. We studied 20 strains of *P. aeruginosa* collected during 2018–2021 by specialists from the Laboratory of Microbiome and Microecology of the Scientific Centre for Family Health and Human Reproduction Problems. The identification of strains was carried out using test systems for differentiating gram-negative non-fermenting bacteria (NEFERMtest 24 Erba Lachema s.r.o., Czech Republic), and confirmed by mass spectrometric analysis and 16S rRNA gene sequencing. Antimicrobial activity was assessed by the degree of inhibition of cell growth in planktonic and biofilm forms (on a flat-bottomed 96-well plastic immunological plate). All clinical isolates of *P. aeruginosa* were biofilm formers, 47.6 % of the isolates were weak biofilm formers, and 52.4 % of the isolates were moderate biofilm formers. Planktonic cells and the forming biofilm of the tested *P. aeruginosa* strains were carbapenems-resistant. Biofilm formation was suppressed in more than 90 % of cases by the agents of the cephalosporin and aminoglycoside groups. Antimicrobial susceptibility of *P. aeruginosa* strains in the formed biofilm was significantly lower ($p < 0.05$). Carbapenems and cephalosporins did not affect the mature biofilms of the tested *P. aeruginosa* strains in more than 60 % of cases. Only non-beta-lactam antibiotics (ciprofloxacin and amikacin) suppressed the growth of planktonic cells and destroyed the mature biofilm. The revealed differences in the effect of the tested antimicrobials on the *P. aeruginosa* strains biofilms correlate with resistance to a number of antibiotics. To prevent biofilm formation in the hospital strains of *P. aeruginosa*, the use of ceftazidime may be recommended, and antimicrobials such as ciprofloxacin and amikacin may be used to affect mature biofilms of *P. aeruginosa*.
Key words: *Pseudomonas aeruginosa*; biofilm formation; antimicrobial drugs; antibiotic resistance.

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Влияние антимикробных препаратов на биопленкообразование *Pseudomonas aeruginosa*

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Аннотация. Синегнойная палочка (*Pseudomonas aeruginosa*) относится к наиболее проблемным патогенам в лечебных учреждениях, что может быть связано со способностью этого микроорганизма существовать в биопленке, которая повышает его устойчивость к антимикробным препаратам, а также распространенность и выживаемость во внешней среде. Цель настоящей работы – оценка чувствительности штаммов *P. aeruginosa*, находящихся в планктонной форме и форме биопленки, к воздействию антимикробных препаратов. Исследовано 20 штаммов *P. aeruginosa* из рабочей коллекции лаборатории микробиома и микроэкологии Научного

центра проблем здоровья семьи и репродукции человека, собранной в течение 2018–2021 гг. Идентификация штаммов проведена с использованием тест-систем для дифференциации грамотрицательных неферментирующих бактерий и подтверждена масс-спектрометрическим анализом и секвенированием гена 16S рРНК. Активность антимикробных препаратов оценивали по степени ингибирования роста клеток бактерий, находящихся в планктонной форме и форме биопленки. Установлено, что все клинические штаммы *P. aeruginosa* были биопленкообразующими, 47.6 % относились к слабообразующим, 52.4 % – к умереннообразующим. Планктонные клетки и формирующаяся биопленка тестируемых штаммов были устойчивы к карбапенемам. Формирование биопленки в более чем 90 % случаев подавляло препараты групп цефалоспоринов и аминогликозидов. Чувствительность к воздействию антимикробных препаратов у штаммов *P. aeruginosa*, находящихся в сформированной биопленке, была значимо ниже ($p < 0.05$). Карбапенемы и цефалоспорины не воздействовали на зрелые биопленки тестируемых штаммов *P. aeruginosa* более чем в 60 % случаев. Только не-бета-лактамные антибиотики (ципрофлоксацин и амикацин) подавляли рост планктонных клеток и разрушали зрелую биопленку. Выявленные различия в действии испытанных препаратов на биопленку штаммов *P. aeruginosa* коррелируют с устойчивостью к целому ряду антибиотиков. Для предупреждения формирования биопленок у больничных штаммов *P. aeruginosa* может быть рекомендовано применение цефтазидима, для воздействия на зрелые биопленки *P. aeruginosa* – антимикробные препараты ципрофлоксацин и амикацин.
Ключевые слова: *Pseudomonas aeruginosa*; биопленкообразование; антимикробные препараты; антибиотикорезистентность.

Introduction

Pseudomonas aeruginosa invariably occupies the leading place among pathogens of nosocomial infections in the Russian Federation and is included in the group of opportunistic bacteria, united by the term ESKAPE (Skleenova et al., 2018). The presence of a wide range of pathogenic factors, genetic flexibility, and the ability to rapidly acquire resistance to different antibiotic groups makes *P. aeruginosa* one of the most problematic pathogens in healthcare settings (Edelstein et al., 2019). Patients with compromised immune systems, eye burns and trauma, and those with internal medical devices are primarily at risk of developing a pseudomonal infection (Diggle, Whiteley, 2020). Pseudomonal infections are particularly dangerous in patients with cystic fibrosis (Kosztołowicz et al., 2020; Scherz et al., 2021).

Treatment of infections caused by *P. aeruginosa* is complicated by the ability of these bacteria to exist in a biofilm, which increases their resistance to antibiotics, their prevalence, and survival ability (de Abreu et al., 2014; Olivares et al., 2020). Destruction of bacterial biofilms formed in the secretions of cystic fibrosis patients was shown to be a serious problem, since diffusion of antibiotics into biofilm structures is poor, and their antibacterial activity can stimulate drug resistance (Kosztołowicz et al., 2020). Classical methods for determining antibiotic sensitivity (broth or agar dilution methods and disc diffusion method) are performed on non-adherent bacteria. The results obtained with these methods cannot predict the therapeutic success of the respective antibiotics against biofilms (Olivares et al., 2020). Currently, there are no guidelines to help clinicians treat biofilm infections, which gives reason for developing routine laboratory methods to determine the sensitivity of biofilm bacteria to antibiotics (Olivares et al., 2020).

According to the experts of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the variety of antipseudomonal antibiotics, sensitivity to which is evaluated under *in vitro* conditions, includes penicillins, cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides and polymyxins[1]. In this regard, we studied the effect of the above groups of antimicrobial agents (AMAs) (ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin and amikacin) on plankton cell growth, forming and mature *P. aeruginosa* biofilm.

The aim of the study was to evaluate the sensitivity of *P. aeruginosa* strains in the planktonic form and in the biofilm form to antimicrobial agents.

Materials and methods

The objects of the study were 20 strains of *P. aeruginosa* with confirmed drug resistance to antimicrobials from the collection of the Laboratory of Microbiome and Microecology of the Scientific Centre for Family Health and Human Reproduction Problems, accumulated during 2018–2021. Type strain *P. aeruginosa* ATCC 27853 (Scientific Centre "Kurchatov Institute" – Research Institute for Genetics and Selection of Industrial Microorganisms) was used as a control.

Hospital strains were isolated from patients from two medical institutions in Irkutsk according to the principle "one patient–one isolate". Eight cultures were obtained from the Irkutsk State Regional Children's Clinical Hospital (Noskova et al., 2020) and 12 cultures were obtained from the City Ivano-Matreninsky Children's Clinical Hospital. Cultures were gathered from patients with different types of diseases (sepsis, acute hematogenous osteomyelitis, peritonitis, pneumonia, etc.) and isolated from oropharynx, liquor, wound, endotracheal tubes, tracheostomy, central venous catheter (14 cultures). A separate group consisted of 6 cultures isolated from the sputum of patients with such a genetic disease as cystic fibrosis (CF).

Identification of *P. aeruginosa* strains. Primary differentiation of *P. aeruginosa* strains was performed by colony morphology, pigment on blood agar, and Gram staining. Biochemical identification of selected cultures was performed using test systems for differentiation of Gram-negative non-

[1] European Committee on Antimicrobial Susceptibility Testing [electronic source]. Clinical breakpoints – breakpoints and guidance. URL: http://www.eucast.org/clinical_breakpoints/ (accessed on: 15 October 2021).

fermenting bacteria NEFERMtest 24 (Erba Lachema s.r.o., Czech Republic), and confirmed by MALDI-TOF using direct protein profiling of nonfermenting microorganisms. Mass spectrometric analysis was performed on the Bruker UltrafleXtreme mass spectrometer (Bruker Daltonics, Germany). Additionally, cultures were identified by a fragment of the ribosomal operon containing the V1–V4 variable regions of the 16S rRNA gene. Full-length 16S rRNA gene fragments of *P. aeruginosa* strains were registered in the international GenBank database under numbers OL616031–OL616034.

To assess the effect of AMA on biofilm formation and destruction of the formed biofilms, antibiotics of the following groups were used: cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, in the form of standard cardboard disks with antimicrobial drugs DI-PLS-50-01, (NICP, Research Centre for Pharmacotherapy, Russia), Hi-Media Laboratories Pvt. Limited (India).

Determination of biofilm formation capacity and biofilm resistance to AMAs using 96-well plastic plates. A 24-hour culture was used for the assay. The inoculum was densified in meat-peptone broth (MPB) to 10^6 CFU/mL. Strains were prepared, culture optical density (OD) was measured, biofilms were stained, and the biofilm formation intensity was determined by measuring the optical density with gentian violet/ethanol extracts, and the biofilm formation coefficient (BFC) was calculated according to the previously described methods (Nemchenko et al., 2020; Grigorova et al., 2021).

Evaluation of the ability of AMA to affect plankton cell growth and biofilm formation. To determine the ability of AMAs to affect plankton cells and the forming biofilm, one AMA disk with the required antibiotic concentration was added to the plate simultaneously with a 24-hour culture: ceftazidime – 10 μg, cefepime – 30 μg, imipenem – 10 μg, meropenem – 10 μg, ciprofloxacin – 5 μg, amikacin – 30 μg. Sterile MPB served as a control. After 30 min, the disks were removed (Tapalskiy, Bilskiy, 2018), the plates were cultured in the thermostat for 24 h, then the experiments were conducted as previously described (Nemchenko et al., 2020; Grigorova et al., 2021).

Evaluation of the ability of AMA to destroy mature biofilms. To determine the ability of AMA to destroy a mature biofilm, plankton cells were removed from the culture plate after 24 h of incubation, washed three times with sterile distilled water, and 150 μL of sterile MPB and one AMA disk were added to each well, including control wells. The disks were removed after 30 min. The plates were incubated for another 24 h. Furthermore, the procedure was similar to that previously described (Nemchenko et al., 2020; Grigorova et al., 2021).

Registration of experimental results. The biofilm formation coefficient (BFC) was calculated after measuring the optical density of the ethanol extract of the stained wells in all plates as the ratio of the optical density of the experiment extract and optical density of the control extract. When the obtained BFC values were less than 2.0, strains were classified as weak biofilm formers, with values of 2.0–3.9, as moderate biofilm formers, and above 3.9, as strong biofilm formers (Nemchenko et al., 2020; Grigorova et al., 2021). The effect coefficient of AMA on forming and mature biofilms was calculated using the formula

$$OD\ BF_{form}/OD\ BF_{without\ AMA} \text{ or } OD\ BF_{mature}/OD\ BF_{without\ AMA},$$

where $OD\ BF_{form}$ or $OD\ BF_{mature}$ is the optical density of the ethanol extract of the biofilm influenced by AMA, $OD\ BF_{without\ AMA}$ is the optical density of the ethanol extract of biofilm cultures without the AMA effect. With a ratio < 0.9, AMA was considered to affect the biofilm; from 0.9 to 1.0, AMA had little effect on the biofilm; from 1.0 and above, AMA had no effect on the biofilm.

The growth of plankton cells in the plate wells was determined as the ratio of the optical density of the bacterial plankton cell suspension after 24 h of cultivation to the initial density; the result was interpreted as previously described (Nemchenko et al., 2020; Grigorova et al., 2021).

Statistical processing of the data was performed using licensed MS Excel 2007 for Windows 7 applications. Non-parametric criteria were used to assess the significance of differences between the two groups according to the level of any criterion: χ^2, Mann–Whitney *U*-criterion. Absolute and relative (percentage) values were calculated for the qualitative variables. The significance level for statistical hypothesis testing (p) was assumed to be 0.05.

Results

It was found that under laboratory conditions without AMA exposure, the planktonic cells of *P. aeruginosa* had a significant growth rate (Table 1). The density of microbial cells increased in 24 h of cultivation more than ten-fold compared to the initial density ($U_{emp} = 0$, differences significant between the initial density and the density after 24 h, Mann–Whitney test).

The OD of *P. aeruginosa* biofilm cultures isolated from sputum in such a severe, genetically determined disease as cystic fibrosis was significantly greater than that of the type strain ($p < 0.01$) and cultures isolated in other diseases (see the Figure). A similar pattern was observed when comparing BFCs. The mean BFC of cystic fibrosis *P. aeruginosa* was 2.79 ± 0.78; *P. aeruginosa* in other diseases was 2.01 ± 0.69; *P. aeruginosa* ATCC 27853 was 1.56.

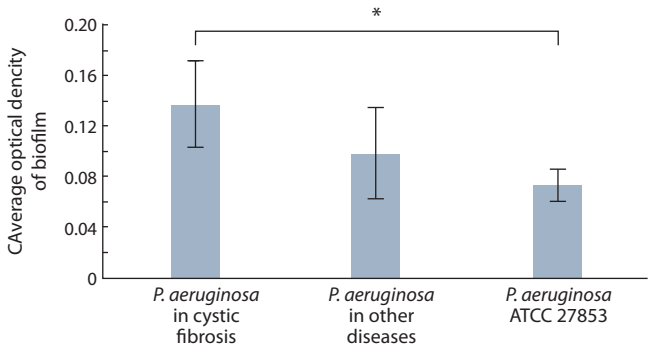
Evaluation of biofilm formation ability by the amount of dye bound to the biofilm showed that the strains studied, including the *P. aeruginosa* ATCC 27853 type strain, were weak biofilm formers in 47.6 %, in 52.4 % of cases were moderate biofilm formers (see Table 1).

A comparison of the optical densities of cultures growing without and under the AMA effect showed that planktonic cells were resistant to AMA imipenem (5 % of sensitive cultures, $U_{emp} = 46$) and meropenem (5 % of sensitive cultures, $U_{emp} = 64.5$; there is a difference between the initial density and the density after 24 h, Mann–Whitney test, $p < 0.05$). The other drugs inhibited the growth of planktonic cells, the most effective were amikacin (60 % of sensitive cultures, $U_{emp} = 180.5$) and ciprofloxacin (50 % of sensitive cultures, $U_{emp} = 191.5$), cefepime affected 40.0 % of cultures ($U_{emp} = 191.5$), and ceftazidime suppressed the growth of *P. aeruginosa* cultures in 35 % of cases ($U_{emp} = 179.0$) (no difference between the initial density and the density after 24 h, Mann–Whitney test, $p > 0.05$).

Table 1. Characterization of the tested *P. aeruginosa* strains by growth rate and biofilm formation intensity

Indicator	Indicator gradation	Strains, %	
Growth rate	No	0	
	Small	0	
	Significant	100	
Biofilm formation intensity	No	0	
	Weak	47.6	
	Moderate	52.4	
Factor	*P. aeruginosa* in cystic fibrosis	*P. aeruginosa* in other diseases	*P. aeruginosa* ATCC 27853
Mean optical density of the biofilm[1]	0.137	0.098	0.073
Mean optical density of MPB (control)	0.047	0.047	0.047
Mean value of BFC	2.79	2.01	1.56

Note. [1] The difference is significant between the optical density of gentian violet/ethanol extracts of the cultures in cystic fibrosis and the optical density of gentian violet/ethanol extracts of *P. aeruginosa* ATCC 27853, U_{emp} = 1 Mann–Whitney test, $p < 0.01$. MPB – meat-peptone broth; BFC – biofilm formation coefficient.



Mean value of biofilm optical density of the tested *P. aeruginosa* strains.

* The difference is significant between the optical density of biofilm cultures in cystic fibrosis and the optical density of biofilm of *P. aeruginosa* ATCC 27853, U_{emp}=1 Mann–Whitney test, $p < 0.01$.

Study of the ability of AMAs to affect the formation and destruction of mature biofilms

The ability of AMAs to affect biofilm formation in *P. aeruginosa* cultures was evaluated using the ratio of the optical density of biofilms exposed to AMAs to the optical density of biofilms without AMA exposure.

The studies showed that not all AMAs prevent biofilm formation (Table 2). Ciprofloxacin had no effect on biofilm formation in 23.8 % of cases, imipenem and meropenem, in 33.3 and 38.1 %, respectively; ceftazidime, cefepime, and amikacin were most effective in suppressing biofilm formation. Significant differences were found only for ceftazidime, which most effectively suppressed biofilm formation, compared with imipenem (χ^2 = 5.62) and meropenem (χ^2 = 7.03) ($p < 0.05$).

The sensitivity of *P. aeruginosa* cells in a mature biofilm to AMA exposure was lower than that of biofilm formation (Mann–Whitney test, difference significant between the optical density of a forming biofilm and a mature biofilm, $p < 0.05$). AMAs ceftazidime, cefepime, imipenem, and meropenem had little or no effect on *P. aeruginosa* biofilms; the $BF_{mature}/BF_{without\ AMA}$ ratio was 0.9 or higher in more than 60 % of cases. Only non-beta-lactam antibiotics, such as amikacin and ciprofloxacin, affected the formed biofilm (Table 3). Comparison of the AMAs effects among themselves showed that amikacin was more effective than ceftazidime ($\chi^2 = 5.01$) and meropenem ($\chi^2 = 10.98$), ciprofloxacin was more effective than meropenem ($\chi^2 = 7.62$).

The BFC of *P. aeruginosa* strains in the formed biofilm was significantly higher than BFC of cultures exposed to AMAs at the stage of biofilm formation, which also confirms the resistance of the mature biofilm. BFC for ceftazidime$_{form/mature}$ U_{emp} = 48.5; cefepime$_{form/mature}$ U_{emp} = 58; imipenem$_{form/mature}$ U_{emp} = 97; amikacin$_{form/mature}$ U_{emp} = 50. There is a difference between the BFC value of the forming and BFC value of the mature biofilm, Mann–Whitney test, $p < 0.01$.

Discussion

The experiment showed that not all AMAs inhibited the growth of planktonic cells of clinical *P. aeruginosa* isolates. Resistance to cephalosporins (ceftazidime and cefepime) was demonstrated by 65 and 60 % of the tested strains, respectively. Resistance to carbapenems (imipenem and meropenem) was observed in almost all isolates. Resistance to non-beta-lactam antibiotics (amikacin and ciprofloxacin) was shown by 40 and 50 % of the strains, respectively. The findings are consistent both with our previous studies (Noskova et al., 2020) and with a multicentre epidemiological study of antibiotic resistance of

Table 2. Ability of AMAs to affect biofilm formation of the tested *P. aeruginosa* strains (absolute value/%)

Antimicrobial agent	Ratio OD BF_{form}/OD $BF_{without\ AMA}$		
	< 0.9	from 0.9 to 1.0	from 1.0 and higher
Ceftazidime[1]	19/95	1/5	–
Cefepimем[2]	18/90	–	2/10
Amikacin[2]	18/90	–	2/10
Ciprofloxacin[2]	15/75	–	5/25
Imipenem[2]	13/65	2/10	5/25
Meropenem[2]	12/60	2/10	6/30

[1] Ceftazidime affects biofilm formation compared to imipenem and meropenem, $p < 0.05$; [2] no difference when comparing the effect between other AMAs, $p > 0.05$; OD BF_{form} – optical density of the forming biofilm under the effect of AMA; OD $BF_{without\ AMA}$ – optical density without AMA exposure; AMAs – antimicrobial agents.

Table 3. Ability of different AMAs to affect the mature biofilm of *P. aeruginosa* strains (absolute value/%)

Antimicrobial agent	Ratio OD BF_{mature}/OD $BF_{without\ AMA}$		
	< 0.9	from 0.9 to 1.0	from 1.0 and higher
Amikacin[1]	12/60	3/15	5/25
Ciprofloxacin[2]	10/50	–	10/50
Ceftazidime	5/25	3/15	12/60
Cefepime	8/40	–	12/60
Imipenem	6/30	–	14/70
Meropenem	2/10	4/20	14/70

[1] Amikacin destroys the mature biofilm compared with ceftazidime ($p = 0.02$) and meropenem ($p < 0.001$); [2] ciprofloxacin destroys the mature biofilm compared to meropenem ($p < 0.05$); OD BF_{mature} – optical density of the mature biofilm under the AMA effect; OD $BF_{without\ AMA}$ – optical density of the biofilm with no AMA effect; AMAs – antimicrobial agents.

nosocomial pathogens ("MARATHON" 2015–2016), which observed an increase in resistance of nosocomial *P. aeruginosa* strains to most AMAs, including carbapenems (Edelstein et al., 2019).

The strains studied, especially those isolated from patients with cystic fibrosis, were biofilm-forming (see Table 1). This served as the basis for us to evaluate the effectiveness of AMAs against the forming biofilm of nosocomial pathogens. The experiment showed that compared to other antibiotics, ceftazidime was the most effective drug inhibiting biofilm formation (see Table 2).

As recent studies show, in addition to classical resistance mechanisms, bacteria are able to withstand exposure to high antibiotic concentrations by exhibiting so-called tolerance (Brauner et al., 2016; Yan, Bassler, 2019). Tolerant bacteria grow more slowly than their non-tolerant counterparts and may avoid death by antibiotic treatment (Brauner et al., 2016). Another form of tolerance, which does not result from inherited mutations but rather from phenotypic differentiation, is commonly referred to as persistence. Time-dependent destruction of the bacterial population by antibiotics shows that actively growing cells die first, while persistent cells die in the second phase at a much lower rate. It is this subset of microorganisms that survives antibiotic exposure and recovers after antibiotic withdrawal (Balaban et al., 2004).

It has been suggested that the ability of biofilms to contain tolerant and persistent cells underlies the difficulties encountered in eliminating biofilms (Lewis, 2012). It is likely that the increased antibiotic tolerance arises from altered biofilm cell physiology. It has been suggested that cells within biofilms are in a stationary phase where the penetration of nutrients and oxygen is limited due to consumption by the cells located peripherally (Yan, Bassler, 2019). The presence of persistent cells can be dangerous in certain groups of patients, such as those with cystic fibrosis, when highly persistent mutants are released after long-term antibiotic treatment (Lewis, 2012).

The studies presented showed that the sensitivity of cells in mature biofilms to AMAs was significantly lower; the antibiotics generally failed to destroy biofilm cultures of *P. aeruginosa*. The BFC of cultures in mature biofilms was higher than that of cultures that were affected by AMA during biofilm formation ($p < 0.01$).

Of all AMAs tested, only non-beta-lactam antibiotics (ciprofloxacin and amikacin) inhibited the growth of plankton cells and destroyed the mature biofilm, which may be related to the mechanism of the effect of different classes of antibiotics. The cells in the biofilm decrease the rate of cell division, making them less sensitive to beta-lactam antibiotics affecting the cell wall, while the effect of ciprofloxacin and amikacin does not require actively dividing cells since it targets transcription and translational processes (Sidorenko et al., 2013; Thieme et al., 2021).

The most effective approach to prevent biofilm formation would be to inhibit the adhesive capacity of cells (Olivares et al., 2020). For example, a study by S. Otani et al. (2018) showed that subinhibitory minimal suppressive concentrations of ceftazidime reduced biofilm mass, suppressed motility and expression of genes involved in bacterial adhesion and *P. aeruginosa* PAO1 matrix production (Otani et al., 2018). Previously, S. Roudashti et al. (2017) observed the effects of cephalosporins in *P. aeruginosa* QS systems providing motility and biofilm formation in these microorganisms (Roudashti et al., 2017). In our study, ceftazidime also showed the highest antibiofilm effect compared with other AMAs. However, the mechanism of biofilm resistance to AMAs is complex, multifactorial, and contradictory. This point is supported by numerous studies that demonstrate that low doses of antimicrobials in the centre of infection can increase the risk of mutagenesis and initiate biofilm formation (Kaplan, 2011; Ciofu et al., 2015; Olivares et al., 2020).

Conclusion

Thus, the study of the effect of AMAs of the groups of cephalosporins, carbapenems, fluoroquinolones and aminoglycosides on the biofilms of the tested hospital *P. aeruginosa* strains showed that the antipseudomonal drugs mainly prevented the formation but did not destroy the already formed biofilm. The significant differences detected in the effect of the tested AMAs both on the mature biofilm of *P. aeruginosa* strains and on the process of its formation to a certain extent correlate with the resistance of this microorganism to a number of antibiotics (Edelstein et al., 2019; Adzhieva et al., 2021). Additional research aimed at detecting tolerant and persistent cells is needed to elucidate the mechanisms involved, which will optimise the overall use of antimicrobials for treating biofilm-related infections (Yan, Bassler, 2019). The use of ceftazidime may be recommended to prevent biofilm formation in the hospital strains of *P. aeruginosa*, and amikacin and ciprofloxacin may be recommended for affecting mature *P. aeruginosa* biofilms.

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