AGRICULTURAL BIOLOGY,
2020, V. 56, Iss. 1, pp. 146-157
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 633.1:631.559.2:579.64

doi: 10.15389/agrobiology.2021.1.146eng
doi: 10.15389/agrobiology.2021.1.146rus

EFFECTS OF NITROGEN-FIXING AND PHOSPHATE-SOLUBILIZING MICROORGANISMS FROM THE FAR EAST AGRICULTURAL SOILS ON THE CEREAL SEED GERMINATION

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The authors declare no conflict of interests
Received November 12, 2019

Abstract

Mineral fertilizers which can significantly increase crop productivity have an adverse effect on the soil and the environment as a whole when used for a long time. Microorganisms, as an alternative to mineral fertilizers, stimulate plant growth and development due to their ability to fix nitrogen, produce siderophores, phytohormones and enzymes, dissolve inaccessible elements of mineral nutrition, suppress plant pathogens, and increase consumption of water and nutrients. However, the effectiveness of such preparations highly depends on factors of a new environment. We believe that bacteria from soils that have long been exposed to various agricultural practices may be good plant stimulants. In the presented study, for the first time, we have isolated local active strains of nitrogen-fixing and phosphate-mobilizing bacteria from soils subjected to 74-year stationary intensive farming in the conditions of the Russian Far East and revealed isolates and their combinations which stimulate wheat and barley seed germination and seedling growth. The aim of the work was to study the plant-stimulating properties of nitrogen-fixing and phosphate-mobilizing bacteria from soils that have been actively exposed to mineral fertilizers for a long time. Bacteria were isolated from soils sampled in October 2015 (the experimental field 8, Federal Research Center for Agrobiotechnology FEB RAS, Ussuriysk, Primorsky Territory, Russia). The seeds of wheat *Triticum aestivum* L. cultivar Primorskaya 50, and barley *Hordeum vurlage* L. cultivar Tikhookeanskii (collection of the Federal Research Center for Agrobiotechnology FEB RAS) were treated. Of 68 bacterial isolates with different cultural and morphological properties, three isolates, the *Acinetobacter* spp. N1, *Azotobacter* spp. N2, and *Clostridium* spp. N3 were nitrogen fixers, and four isolates, the *Serratia* spp. P6, *Bacillus* spp. P7), *Arthrobacter* spp. P8, and *Pantoea* spp. P19 were phosphate-mobilizing bacteria. Tests with the monocultures of nitrogen-fixing and phosphate-mobilizing isolates and their different binary compositions showed a 13-51 % increase ($p \leq 0.05$) in wheat seed germination energy and 15-54 % increase ($p \leq 0.05$) in barley seed germination energy compared to the untreated control. Laboratory germination of wheat seeds increased by 2-32 %, barley seeds by 7-30 % compared to untreated control. The barley seedlings were 1.8 times longer, and the roots were 2.7 times longer. Th binary combination N2P19, P6P19, and P8P19 caused the highest height of seedlings (120-140 mm, $p \leq 0.05$), and with P6P7, N2P19, and P6P19 the roots were the longest (120-130 mm, $p \leq 0.05$). These results allow us to conclude that short-term soaking seeds in the suspensions of the tested nitrogen-fixing and phosphate-mobilizing isolates improves seed germination energy and laboratory germination, and increases shoot and root length. Binary bacterial compositions have a greater effect on seed germination than monocultures. The strains N1 (*Acinetobacter* spp.), N2 (*Azotobacter* spp.), and P19 (*Pantoea* spp.) are the best stimulants. Species-specific differences in plant response to the treatment is probably due to lack of genetic, biochemical and physiological complementarities between specific plant species and the bacteria.

Keywords: biological fertilizers, diazotrophs, phosphate-mobilizing bacteria, soil, long-term chemicalization, *Triticum aestivum* L., wheat, *Hordeum vurlage* L., barley, seeds, germination energy,

laboratory germination, seedlings

Intensive exploitation of agricultural land is accompanied by the constant use of high doses of mineral fertilizers [1, 2], which can significantly increase productivity but with the long-term use leads to a decrease in the quality of crop production, environmental pollution, and violation of natural mechanisms of soil restoration [3-5]. Therefore, at present, mono, binary and multicomponent microbial preparations are increasingly used instead of mineral fertilizers. Unlike mineral fertilizers, they have some advantages: they do not pollute the environment, they are harmless to humans and animals, not phytotoxic, and do not have mutagenic activity since they are strains of natural soil microorganisms.

Microorganisms that serve as the basis of biological products, by entering into close interactions with the host plant and other participants in the microbiocenosis, stimulate the growth and development of plants by using direct and indirect mechanisms: the fixation of atmospheric nitrogen, the production of siderophores, phytohormones, and enzymes, the dissolution of inaccessible elements of mineral nutrition [6-8]. Some strains inhibit the development of phytopathogenic microorganisms living in the soil due to the production of antimicrobial metabolites [9]. The introduction of promising bacterial inoculates can reduce the phytotoxicity of soil contaminated with heavy metals [10, 11]. Through treatment with microbial preparations, it is possible to increase the stress resistance of plants in drought conditions by increasing the absorption of moisture and nutrients, as well as the production of exopolysaccharides [12, 13]. However, the effectiveness of the introduced preparations strongly depends on the factors of the new environment and the ability of the introduced microorganisms to survive among the indigenous representatives of the soil microflora [14].

The way out of this situation can be the creation of plant growth stimulants based on bacteria isolated from soils that have been exposed to various agricultural practices for a long time. Microorganisms that have preserved their viability in conditions of intensive farming may have high adaptability to the soil and climatic conditions of cultivated soils.

In this study, the authors first isolated local active strains of nitrogen-fixing and phosphate-mobilizing bacteria from the soils of long-term stationary experience in intensive farming in the Russian Far East and proved the possibility of creating plant growth stimulants based on such bacteria.

The work aims to study the effect of nitrogen-fixing and phosphate-mobilizing bacteria isolated from soils that have been exposed to active chemicalization for a long time on the germination of cereal seeds for the subsequent development of biological preparations of plant growth stimulants.

Materials and methods. Samples of agricultural soils involved in a long-term experiment (74 years) on fertilization were taken in October 2015 (the experimental field 8, the Federal Research Center for Agrobiotechnology of the Far Eastern Branch of the Russian Academy of Sciences) at a depth of 5-15 cm with sterile instruments and placed in sterile dishes.

The influence of soil microorganisms on seed germination was evaluated on wheat (*Triticum aestivum* L.) of the Primorskaya 50 cultivar and barley (*Hordeum vurlage* L.) of the Tikhookeansky cultivar (obtained from the collection of the Federal Research Center for Agrobiotechnology, the Far Eastern Branch RAS).

Strains of nitrogen-fixing and phosphate-mobilizing bacteria were isolated using standard methods used in soil microbiology [15]. A 1:10 soil suspension (10 g of soil and 90 ml of water) was used to prepare a sequence of serial dilutions with inoculation on agar media: nitrogen-fixing microorganisms were isolated on Ashby's Glucose Agar (HiMedia, India), and phosphate-mobilizing microorganisms were isolated on a selective medium with tricalcium phosphate [16].

The strains of microorganisms of greatest interest were previously identified as representatives of *Acinetobacter* spp. (strain N1), *Azotobacter* spp. (strain N2), *Clostridium* spp. (strain N3), *Serratia* spp. (strain P6), *Bacillus* spp. (strain P7), *Arthrobacter* spp. (strain P8), and *Pantoea* spp. (strain P19).

Working suspensions of nitrogen-fixing and phosphate-immobilizing bacteria were obtained by cultivation in a nutrient broth (nutrient broth, Federal Budget Institution of Science State Research Center for Applied Microbiology and Biotechnology, Russia). The number was taken into account using a photoelectric colorimeter AP-101 (APEL Co., Ltd., Japan) at $\lambda = 600$ nm, and for calibration, the count of microbial cells in the Goryaev-Tom chamber (Axioscop 40 microscope, Carl Zeiss, Germany) was used [17]. After determining the initial counts of bacteria, a saline solution was used to dilute the suspension of microbial cells to a working concentration of 1×10^7/ml.

The effect of isolates of nitrogen-fixing and phosphate-mobilizing bacteria on the germination of cereal seeds was evaluated by the synergy effect in binary compositions of monocultures. A sterile nutrient broth was used as a comparison control. Tap water was used as the experiment control.

Plant seeds (100 of each cultivar) were soaked for 30 minutes in the tested monoculture or binary composition of the studied bacteria. Then, the seeds were laid out in 10 pieces on filter paper moistened with sterile water, in sterile Petri dishes, and germinated at 23 °C. On day 3, the germination energy was determined, and on day 7, the laboratory germination rate, seedling length, and root length were determined [18]. The experiment was carried out in a 3-fold replication.

Statistical data processing (calculation of the mean values *M*, the standard error of the mean values ±SEM, parametric comparison by the Student's *t*-test) was performed using the program SPSS v. 11.5 for Windows (https://spss.software.informer.com/11.5/). The differences were considered statistically significant at $p \leq 0.05$.

Results. The soil is a system of many different micro-and mesic environments, providing conditions for the development of a wide variety of soil microorganisms [19]. The ability to stimulate the growth and development of plants was noted in a large number of microorganisms isolated from soils, including representatives of the genera *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Pantoea* spp., *Enterobacter* [2, 20-22]. These microorganisms can synthesize vitamins, amino acids, polyhydroxy butyrate, phytohormones, siderophores, antibiotics, and enzymes, as well as fix molecular nitrogen and mineralize phosphates and other elements [23].

From the naturally formed microbial associations of soil fields of a long-term (74 years) stationary experiment in chemization (Federal Research Center for Agrobiotechnology of the Far Eastern Branch of the Russian Academy of Sciences), the authors isolated 68 bacterial strains with different culture-based and morphological properties. Based on these isolates, the authors created a collection of nitrogen-fixing and phosphate-mobilizing bacteria, screening of which by target characteristics (nitrogen fixation and phosphate mobilization) revealed seven of the most active strains, which were used in further experiments (Table 1).

1. Cultural and morphological properties of bacterial isolates from soils of the 74-year stationary chemicalization experiment (Federal Research Center for Agrobiotechnology of the Far Eastern Branch of the Russian Academy of Sciences, Primorye Territory, 2015)

Trait	Nitrogen-fixing bacteria			Phosphorus-mobilizing bacteria			
	strain N1	strain N2	strain N3	strain P6	strain P7	strain P8	strain P19
Colony description:							
size, mm	< 1	5	3	< 1	1	< 1	2
surface	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy
color	Yellow	Yellowish	Yellowish	White	White	White	Yellow
transparency	–	+	+	–	–	–	–
edge	Smooth	Laciniated	Smooth	Wavy	Laciniate	Smooth	Smooth
profile	Convex	Convex	Convex	Convex	Convex	Convex	Convex
center	+	–	–	–	–	–	–
surface	Crateriform	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
structure	Smooth	Homogeneous	Fine-grained	Homogeneous	Fine-grained	Fine-grained	Homogeneous
consistency	Easy to remove	Mucosa	Mucosa	Easy to remove	Easy to remove	Easy to remove	Easy to remove
Cell description:							
Gram staining	–	–	–	–	–	–	–
shape	Rods	Rods	Rods	Short rods	Short rods	Short rods	Rods
size, µm	1×0.5	0.8×0.4	0.8×0.3	1×1.5	1.2×1.8	1.2×1.8	1×0.4

N o t e. The strains were previously identified as *Acinetobacter* spp. (N1), *Azotobacter* spp. (N2), *Clostridium* spp. (N3), *Serratia* spp. (P6), *Bacillus* spp. (P7), *Arthrobacter* spp. (P8), and *Pantoea* spp. (P19); «–» and «+» — the absence or presence of the trait, respectively.

In this experiment, working suspensions of microorganisms were combined in a ratio of 1:1, resulting in 28 binary compositions and monocultures of nitrogen-fixing and phosphate-mobilizing bacteria (Table 2).

2. Scheme of combinations of nitrogen-fixing and phosphate-mobilizing bacteria used in the experiment

Strain	Nitrogen-fixing bacteria			Phosphate-mobilizing bacteria			
	N1	N2	N3	P6	P7	P8	P19
Nitrogen-fixing bacteria:							
N1	+	+	+	+	+	+	+
N2	–	+	+	+	+	+	+
N3	–	–	+	+	+	+	+
Phosphate-mobilizing bacteria:							
P6	–	–	–	+	+	+	+
P7	–	–	–	–	+	+	+
P8	–	–	–	–	–	+	+
P19	–	–	–	–	–	–	+

N o t e. The strains were previously identified as *Acinetobacter* spp. (N1), *Azotobacter* spp. (N2), *Clostridium* spp. (N3), *Serratia* spp. (P6), *Bacillus* spp. (P7), *Arthrobacter* spp. (P8), and *Pantoea* spp. (P19).

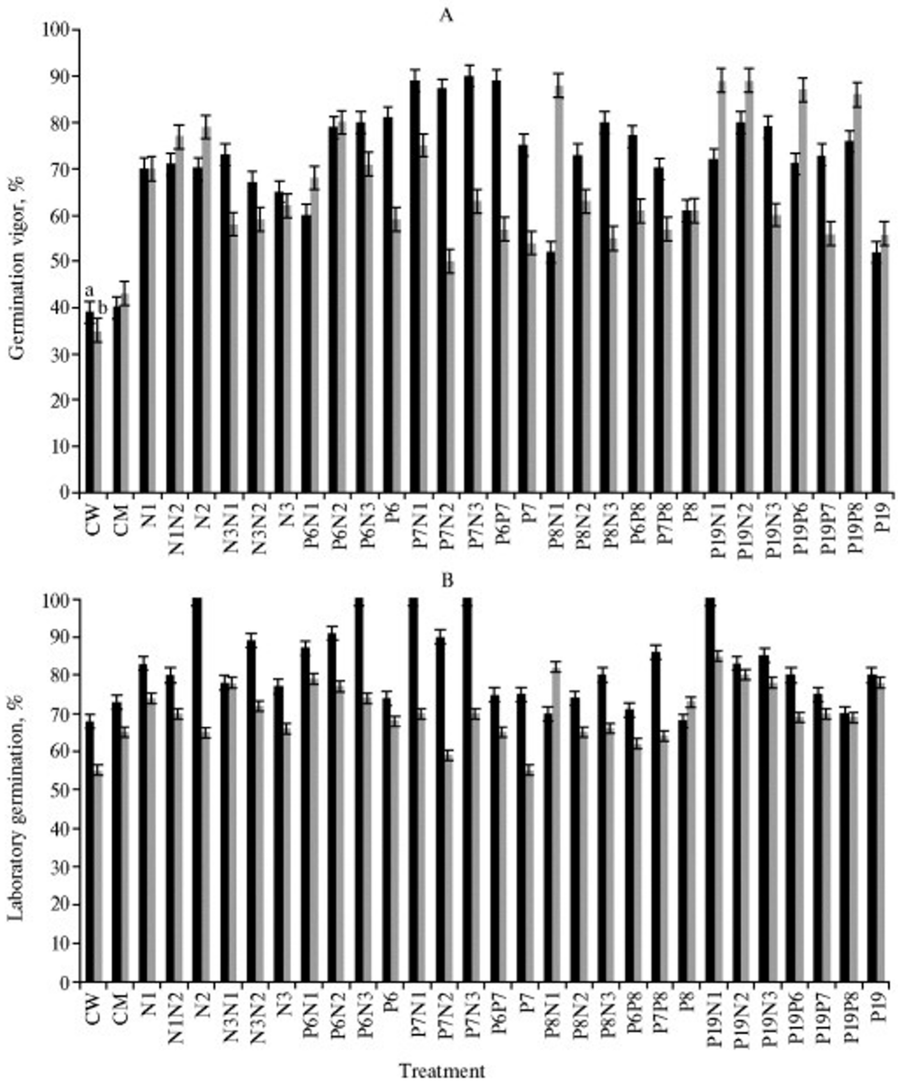

Fig. 1. Seed germination energy (A) **and laboratory germination** (B) **in wheat (*Triticum aestivum* L.) cv. Primorskaya 50** (a) **and barley (*Hordeum vurlage* L.) cv. Tikhookeansky** (b) **when treated with cultures of bacterial isolates:** N1 (*Acinetobacter* spp.), N2 (*Azotobacter* spp.), N3 (*Clostridium* spp.) (nitrogen-fixing),

P6 (*Serratia* spp.), P7 (*Bacillus* spp.), P8 (*Arthrobacter* spp.), P19 (*Pantoea* spp.) (phosphate-mobilizing); CW — control (water), CM — control (nutrient medium) ($N = 3$, $M \pm$SEM). For all treatments, the differences are statistically significant at $p \leq 0.05$.

It was found that as a result of the treatment of seed material by the studied bacteria, the germination energy and laboratory germination rate varied depending on the experiment option and the type of plant. Thus, in wheat and barley, the seed germination energy in the control was 39 and 35%, respectively, in the comparison control – 40 and 43%, while when treated with nitrogen-fixing and phosphate-mobilizing bacteria, this indicator increased in wheat by 13-51%, in barley – by 15-54% compared to the untreated control. Wheat seeds had the highest germination energy in options N1P7, N3P7, and P7P6, while barley seeds had the highest germination energy in options N1P8, N1P19, and N2P19 (Fig. 1).

Laboratory seed germination in wheat and barley in the untreated control was 68 and 55%, respectively, in the comparison control 73 and 65%, when treated with nitrogen-fixing and phosphate-mobilizing bacteria, the laboratory seed germination increased by 2-32% and 7-30%, respectively, compared to the control without treatment. Full germination (100%) of wheat seeds was observed when using binary combinations of N3P6, N3P7, N1P19, as well as monoculture N2. The highest values of the laboratory seed germination index (80-85%) in barley were observed in the options N2P19, N1P8, and N1P19 (see Fig. 1).

The study of the influence of the microorganisms under investigation on the growth and development of wheat seedlings revealed the following significant ($p \leq 0.05$) morphometric changes relative to the control options (the difference between the controls was insignificant and lied in the range of the experimental error): an increase in the seedling length by 20.9%, in the root length by 83.7%. The maximum values of the seedling length were recorded for N2N3 (86.4 mm), N1P8 (87.4 mm), and N2P19 (88.3 mm) (Fig. 2), of the root length for N1N2 (165.4 mm), N2 (159.1 mm), and P19N3 (148.0 mm). In some treatments, the seedling length decreased by 5.2%, the root length by 17.4% compared to the controls ($p \leq 0.05$).

Treatment of barley seeds with strains of nitrogen-fixing and phosphate-mobilizing bacteria in most options also led to an increase in the seedling length by 1.8 times, and the root length by 2.7 times ($p \leq 0.05$). The greatest seedling length was observed in the options treated with combinations of bacteria N2P19 (140.0 mm), P6P19 (121.0 mm), P8P19 (120.0 mm), the root length was maximum when seeds were treated with P6P7 (119.2 mm), N2P19 (121.1 mm), and P6P19 (132.8 mm) suspensions (see Fig. 2). As with wheat, barley in some cases showed a decrease in morphometric characteristics: seedling length by 36.7%, root length by 46.7% compared to the control options ($p \leq 0.05$). It is interesting to note that the N3P7 combination, the treatment with which positively affected the germination energy and laboratory germination of seeds (especially in wheat), contributed to a reduction in the seedling and root length in both barley and wheat. In different cultures, the responsiveness of germinating seeds to treatment with the same combination of bacteria was not the same. Thus, a pair of P6P7 isolates significantly stimulated the growth of seedlings and roots in barley but did not significantly affect these characteristics in wheat.

For the N2P8 combination, the effect was reversed: in wheat germs, the length of the seedling and root increased markedly, while in barley seedlings – slightly and even in some cases decreased compared to the control ($p \leq 0.05$). Thus, we noted an increase in the seedling and root length in wheat compared to to both controls, while compared to one control (nutrient broth) it was longer (seedlings by 12 mm, roots by 50 mm), compared to the other (water) shorter (seedlings by 10 mm, roots by 47 mm). In barley, these indicators generally decreased compared to both controls, but not equally: in comparison with the water, a 6 mm decrease in the seedling length and a 3 mm increase in the root length occurred and as compared to nutrient broth the seedlings were 4 mm longer and the roots were 3 mm longer.

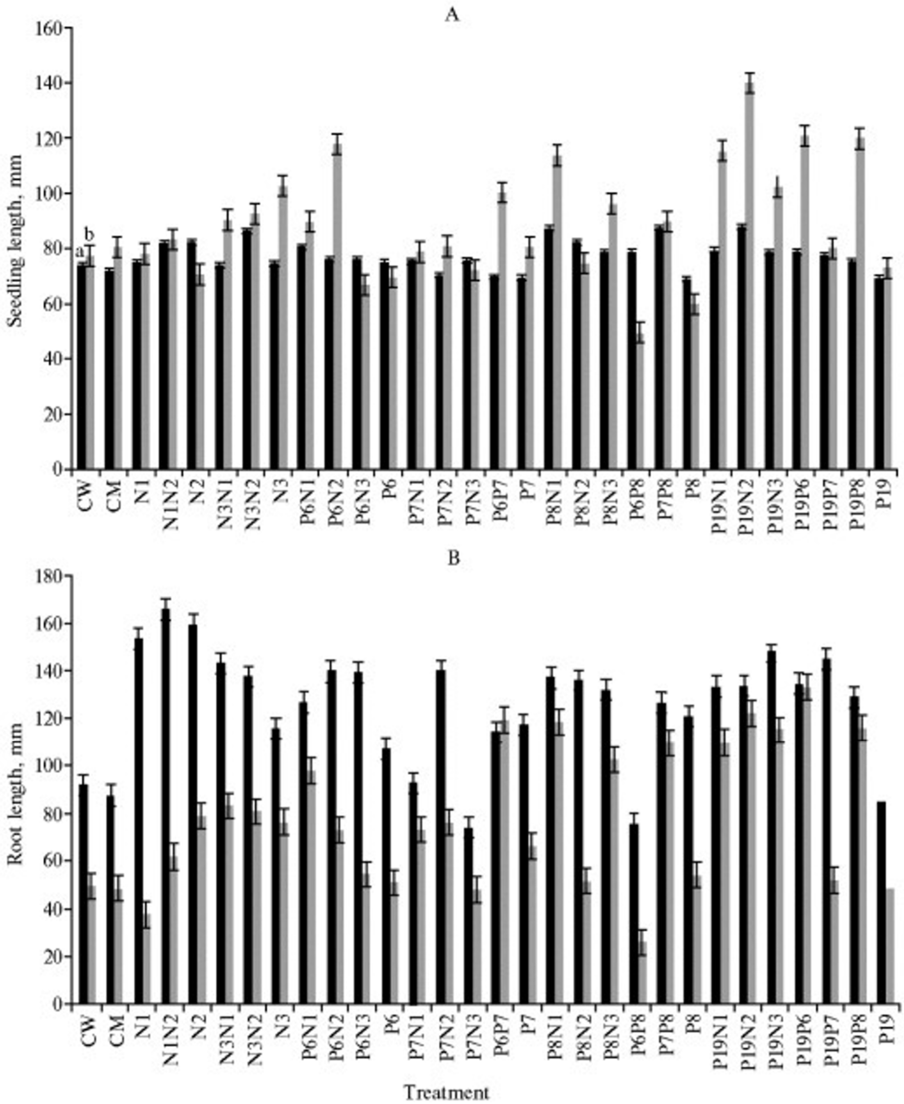

Fig. 2. Seedling length (A) **and root length** (B) **in wheat in wheat (*Triticum aestivum* L.) cv. Primorskaya 50** (a) **and barley (*Hordeum vurlage* L.) cv. Tikhookeansky** (b) **when treated with cultures of bacterial isolates:** N1 (*Acinetobacter* spp.), N2 (*Azotobacter* spp.), N3 (*Clostridium* spp.) ((nitrogen-fixing), P6 (*Serratia* spp.), P7 (*Bacillus* spp.), P8 (*Arthrobacter* spp.), P19 (*Pantoea* spp.) (phosphate-mobilizing); CW — control (water), CM — control (nutrient medium) ($N = 3$, $M \pm$SEM, lab test). For all treatments, the differences are statistically significant at $p \leq 0.05$.

Germination energy and seed germination are important indicators of plant competitiveness, which affects their development and yield [24]. In our experiments, the treatment of wheat and barley seeds with suspensions of the studied nitrogen-fixing and phosphate-mobilizing isolates contributed to a significant and reliable ($p \leq 0.05$) increase in germination energy and laboratory germination. The most expressed stimulating effect was due to combinations of bacteria, including strains N1, N2, P7, and P19. Hahn et al. [22] treated rice (*Oryza sativa* L.) seeds under sterile conditions with liquid cultures containing nitrogen-fixing bacteria of the genera *Burkholderia* and *Mesorhizobium*. Experiments have shown [22] that the initial germination, calculated as the percentage of seeds sprouted on day 3, from the number of seeds sprouted on day 6, in the options with treatment increased by 20-38% compared to the control. A significant increase in laboratory germination of corn was observed with the use of nitrogen-fixing bacteria of the genera *Pseudomonas*, *Bacillus*, *Enterobacter,* and *Pantoea* [21].

Laboratory germination and field germination may differ. For example, in the experiments of Batool and Iqbal [25], the increase in the germination of decontaminated wheat seeds treated with various strains of phosphate-solubilizing bacteria and germinated in Petri dishes concerning the control was 65-90%, while in the field when grown in vegetation vessels, the indicator increased by 20-80% compared to the control. Such discrepancies can be associated with sharply different environmental conditions and, as a result, with a change in the character of interactions in the plant—microorganism system. At the same time, fairly stable interactions are also possible: all the phosphate-solubilizing bacteria studied in the experiment had a positive effect on the development of wheat plants and were recommended by the authors in a monoculture or a combination as an alternative to mineral fertilizers [25]. It has been reported that inoculation of seeds with biological preparations containing live microorganisms has a positive effect on germination, while the effect depends on the weather conditions accompanying the beginning of the vegetation period [26]. It is evident that to ensure a positive result in microbial-plant interaction, the introduced microorganisms must not inhibit the growth and development of the partner plant. The stimulating or inhibitory effect of bacteria on the development of plants of barley *Hordeum vulgare* L., spring wheat *Triticum aestivum* L., rye *Secale cereale* could be detected even at the seed germination stage [2]. According to the literature, the effect of inhibiting plant development can be associated with a high concentration of the phytohormone indolyl-3-acetic acid, which in low concentrations stimulates growth [27]. Under laboratory conditions, the inhibitory effect of associative diazotrophs on germinating seeds could be manifested due to the absence or lack of nutrients in the environment necessary for the growth of this group of microorganisms. As it is known, rhizosphere bacteria consume mainly the metabolic products released by germinating seeds, but in the absence of photosynthesis, their number is small [20].

The combinations of the studied bacteria in most options contributed to an increase in the seedling length and the root length in the germs of both plant species to varying degrees ($p \leq 0.05$). An increase in the root length was observed when wheat seeds were treated with bacteria of the genus *Bacillus* [28]. The most expressed stimulating effect on these morphometric characteristics was exerted by combinations that included strains P19, N1, N2, and binary compositions that included both nitrogen-fixing and phosphate-mobilizing bacteria showed a generally more expressed effect than monocultures of the same microorganisms. Similar observations were made by Widawati and Suliasih [29], by studying the effect of bacteria of the genera *Azotobacter*, *Azospirillum*, and *Bacillus* on the germination of sorghum seeds S*orghum bicolor* L., they found that the indicators of germination energy, seedling and root length decreased after inoculation with monocultures even relative to the control (the exception was the option with the treatment with *Azospirillum lipoferum*). At the same time, simultaneous treatment with nitrogen-fixing bacteria of the genus *Rhizobium* and phosphate-solubilizing bacteria of the genus *Pseudomonas* contributed to an increase in the mass of roots, seedlings, and wheat yield [30].

Let us note that the effect of the bacterial inoculates studied by the authors on seed germination depended on the plant type. Thus, in the N1P8 option, the treatment did not have a noticeable effect on wheat seeds, but in the experiment with barley, the same binary composition provided the maximum values of germination energy and laboratory germination of seeds. A similar pattern was observed for the N1P7 combination but, in this case, the greatest stimulating effect was observed for wheat. It was reported [31] that barley yield was more responsive to treatment with the drug Rizoagrin than wheat. Moreover, for soybeans, varietal differences were noted when treated with *Bacillus subtilis* strains. It may be due to the synthesis of certain metabolites, the effectiveness of which directly depends on the plant genotype and its life cycle [32].

Thus, it was found that the treatment of wheat and barley seeds with suspensions of isolates of nitrogen-fixing and phosphate-mobilizing bacteria isolated from soils subjected to long-term (74 years) systematic treatment with mineral fertilizers contributed to a significant increase in the germination energy and laboratory germination of seeds, the seedling and root length. It was shown that the germination energy of wheat seeds in the options treated with nitrogen-fixing and phosphate-mobilizing bacteria increased by an average of 32% (from 13 to 51%) ($p \leq 0.05$), and of barley seeds by an average of 34.5% (from 15 to 54%) ($p \leq 0.05$) compared to the untreated control. At the same time, the laboratory germination of wheat seeds in the options with treatment with bacteria capable of nitrogen fixation and phosphate immobilization increased by 2-32% ($p \leq 0.05$), and barley seeds by 7-30% ($p \leq 0.05$) compared to the untreated control. The greatest seedling length was noted when using combinations of N2P19, P6P19, and P8P19, root length when using P6P7, N2P19, and P6P19. Binary bacterial compositions had a greater effect on the germination of plant seeds than monocultures. The combinations that included nitrogen-fixing strains N1 (*Acinetobacter* spp.), N2 (*Azotobacter* spp.), and phosphate-mobilizing strain P19 (*Pantoea* spp.) showed the most pronounced stimulating effect. Also, the differences in the responsiveness of plants to treatment depending on the species were observed, probably due to the lack of genetic and physiological and biochemical complementarity between a particular plant species and the studied isolate or their combination. Later, we plan to study the effectiveness of these bacterial inoculates in vegetation experiments and the field.

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