

Article

Plant Growth-Promoting and Antifungal Activity of *Bacillus* spp. Isolated from Wild Wheat *Aegilops cylindrica* Against *Fusarium culmorum* in Cultivated Wheat

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Abstract

Wild grasses such as *Aegilops cylindrica* are a promising source of epiphytic bacteria with plant growth-promoting (PGP) properties and those that contribute to biocontrol. Two bacterial strains from the phyllosphere of *A. cylindrica* were isolated and identified as *Bacillus mojavensis* WP4 and *Bacillus siamensis* WS6. Both strains produced auxins (1.56–5.09 µg/mL) and siderophores and dissolved phosphates. They inhibited the growth of *F. culmorum* in vitro and increased the biomass of wheat seedlings by up to 2.9 times, although the effects were variety-specific (Tulaykovskaya 10 variety). Neither strain exhibited multiple drug resistance. *B. mojavensis* WP4 and *B. siamensis* WS6 are multifunctional strains that stimulate plant growth and exhibit biocontrol activity against *F. culmorum* during the germination stage. But visible protection of young plants (not seeds) was not observed; however, bacterial treatment restored photosystem II activity (Fv/Fm and Y(II)) in infected plants. Microscopic examination confirmed root colonization. Future research should focus, on the one hand, on conducting experiments in greenhouses and under field conditions, and on the other hand, on investigating the molecular mechanisms underlying the interaction between the bacterium (*Bacillus* sp. strain), the plant (wheat), and the phytopathogen (*F. culmorum*).

Keywords: PGP-bacteria; wheat; *Fusarium culmorum*; phytopathogen protection; photosystem II

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1. Introduction

Wheat (*Triticum aestivum* L.) is the third-largest food crop grown worldwide. According to the FAO (Food and Agriculture Organization of the United Nations), the wheat crop area in 2020 was 220 million hectares—the largest area of any agricultural crop (maize ranks second at 200 million hectares, and rice ranks third at 164 million hectares) [1].

Wheat production faces several abiotic and biotic stresses. Among fungal plant diseases, fusarium wilt is one of the most devastating, affecting wheat growth, yield, and grain quality. The fungus *Fusarium culmorum* causes a complex of diseases in wheat, collectively known as fusarium wilt. Depending on the part of the plant affected, two main forms are distinguished: Fusarium head blight and root rot (Fusarium crown rot, foot rot, or root rot). Thus, in susceptible wheat plants, all plant parts are susceptible to the disease, and they can be infected by the pathogen at all stages of growth and development [2]. Thus, the fungus causes significant damage to the plant and is the cause of significant yield losses in many wheat-growing regions [3].

One of the current trends in modern agriculture is the transition to organic farming, which involves minimizing, or even completely eliminating, the use of synthetic fertilizers, pesticides, and other chemical compounds. This is due not only to stricter legal restrictions on the use of agrochemicals, but also to a growing awareness of the negative impact of their use on soil microbiota, human health, and the development of resistant strains of plant pathogens. Against this backdrop, the development of biological control agents aimed at preventing plant diseases, improving plant nutrition, and promoting growth is increasingly important [4]. One aspect of this activity is the search for microorganisms capable of preventing the growth of phytopathogens of both bacterial and fungal origin. Microorganisms and their secondary metabolites, which exhibit antibacterial and fungicidal activity, act as biocontrol agents for the development of phytopathogenic bacteria and fungi [5].

In recent years, many antagonistic microorganisms have been used for the biological control of fungal diseases in agricultural crops. Screening and isolation of an increasing number of microbial strains that serve as broad-spectrum biological control agents are ongoing. Bacteria from genera such as *Bacillus*, *Streptomyces*, and *Pseudomonas* are frequently used for the biological control of certain fungal diseases in plant protection [6,7]. A number of studies have also shown that some of these bacteria can stimulate plant growth through direct and indirect mechanisms, including the production of phytohormones, phosphate solubilization, and the synthesis of siderophores [8]. However, despite their obvious advantages, the introduction of bacterial biopreparations into agricultural practice is hampered by insufficient study of effective strains from alternative ecological niches, as well as the need to find new isolates with high colonization capacity and stable antagonistic activity in various soil and climatic conditions. This is evidenced by the fact that biocontrol products currently account for only about 1% of global agrochemical sales, highlighting the urgent need to identify and develop new agents and methods for biological crop protection [9].

The phyllosphere, the aerial surface of plants, is the main habitat for microorganisms interacting with leafy phytopathogens and their spores. While rhizospheric and endophytic bacteria have been extensively studied as sources of biological control agents, epiphytic communities remain relatively poorly understood, especially those associated with wild cereal-related plants [10,11].

To enhance the beneficial effects of biocontrol agents, we propose the isolation of active microorganisms from the phyllosphere of wild relatives of wheat. Unlike the rhizosphere, the phyllosphere is characterized by extreme habitat conditions: bacteria here are exposed to intense ultraviolet radiation, sharp daily temperature fluctuations, water and nutrient deficiencies, and high osmotic stress. This strong selective pressure often leads phyllosphere bacteria to develop enhanced adhesion mechanisms, extracellular polymeric substance (EPS) production, and efficient quorum sensing systems, allowing them to form stable biofilms [12] on waxy and hydrophobic leaf surfaces. We hypothesize that these adaptive strategies not only ensure the survival of microorganisms in the phyllosphere but may also enhance their colonization capacity

upon subsequent contact with the root system, making them more competitive in the rhizosphere compared to typical soil saprophytes. Despite this, the functional potential of bacteria isolated from leaf surfaces as agents for protecting cereal crops from phytopathogens remains virtually unexplored, which underscores the novelty of our approach.

Wild cereals have evolved effective mechanisms of resistance to pathogens and stress, and thus can serve as a source of “beneficial” bacteria that suppress the growth of phytopathogens (causative agents of late blight, rhizoctonia, scab, etc.), stimulate root growth, increase the availability of nutrients from the soil, and induce systemic plant resistance to diseases [10,11]. One such wild relative is *Aegilops cylindrica* Host (cylindrical wheat), an annual grass of the family Poaceae. It typically grows on open rocky and fine-grained slopes, sands, pebbles, roadsides, wastelands, and occasionally in weedy meadows and steppes, at altitudes ranging from 100 to 2000 m above sea level, and is considered a typical weed of the steppe zone [13].

In this study, we isolated seven bacterial strains from the phyllosphere of wild *Aegilops cylindrica* that exhibited both antifungal activity and plant growth-promoting (PGP) traits. Based on their antagonism against *Fusarium culmorum* and the highest PGP characteristics, two strains (WP4 and WS6) were selected and identified. We then evaluated their comparative ability to enhance seed germination, promote plant growth, and protect two wheat varieties (Tulaikovskaya 10 and Darkhan 212) from *F. culmorum* under controlled conditions.

2. Materials and Methods

2.1. Isolation and Cultivation of New Isolates

Microorganisms were isolated from phyllospheric part of *Aegilops cylindrica* growing in the vicinity of the Moscow region, Pushchino (54.847078, 37.628976). Plants were collected in the summer during the active growth period. The upper green parts (leaves and stems) were cut from 10 individual plants with sterile scissors. Each plant sample was processed separately. The plant material was placed in 50 mL of sterile tap water and vigorously shaken for 30 min to obtain a washing suspension containing epiphytic microorganisms. The resulting aqueous solution was diluted 10, 100, and 1000 times, and 1 mL of each dilution was distributed onto a Petri dish with nutrient agar LB (g/L): peptone—10, yeast extract—5, NaCl—10; agar—20 (Amresco, Richmond, Canada)). All bacterial isolates were cultured in LB medium or in LB with the addition of agar at 28 °C for 1 day.

To confirm the sterility of the rinsing water and the absence of external contamination, negative control plates (noninvasive LB agar) were incubated next to the experimental plates under the same conditions. As an additional negative control, sterile tap water used for washing was also applied to LB agar. All procedures were performed in triplicate for each of the 10 plants (biological repeats).

The culture liquid for processing seeds and plants was used at a cell concentration of 10^4 CFU/mL.

2.2. Cultivation of *Fusarium culmorum* VKM F-2303

In this study, *Fusarium culmorum* VKM F-2303 was used as a model phytopathogen. The fungi were cultivated on potato dextrose agar at 25 °C for 7–8 days. Spores were obtained by washing from the surface of the mycelia with sterile distilled water and filtered through a sterile filter. The filtrate was centrifuged at 6000 rpm for 15 min. The precipitate was washed three times with sterile distilled water and centrifuged at 10,000

rpm for 2 min. To infect plants, a 100-microliter suspension with a spore concentration of 20,000 to 30,000 spores per milliliter is used.

The concentration of the resulting suspension of each strain is calculated in a Goryaev chamber (analogue of the chamber Helber [15]), where a certain volume of suspension is distributed over a certain area of glass with an applied grid, and by counting cells per unit volume, the number of cells in the entire volume of the suspension can be determined [15]. The calculation formula is: $X = A \cdot 5 \cdot 1000$, where X is the number of spores in 1 mL of suspension, A is the total number of spores in 50 squares of the chamber. The spore concentration is determined as the average of three independent counts: $X_{avg} = (X1 + X2 + X3)/3$.

2.3. Phylogenetic Analysis

Genomic DNA was isolated from cells using the ExtractDNA Blood & Cells Kit (Evrogen, Moscow, Russia) according to the manufacturer's recommendation. The 16S rRNA gene was amplified by PCR using primers universal for 16S rRNA prokaryotes: 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-TACGGYTACCTTGTTACGACTT3'). The amplified DNA was purified using the Cleanup Standard Kit (Evrogen, Russia). Sequencing of PCR DNA fragments was performed on an Applied Biosystems 3130 Genetic Analyzer automatic sequencer (Thermo Fisher Scientific, Waltham, USA).

The 16S rRNA gene of the WP4 and WS6 strain is deposited in the NCBI GenBank database under the accession number PZ538734 and PZ538736 respectively. Primary phylogenetic screening of the obtained sequences was performed using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast>) and the EzBioCloud database (www.ezbiocloud.net). For phylogenetic analysis, 16S rRNA gene sequences were taken from the GenBank database (www.ncbi.nlm.nih.gov). The nucleotide sequences of the 16S rRNA gene obtained for the strains were manually aligned with the sequences of reference strains of the nearest microorganisms. Phylogenetic tree constructed using partial 16S rRNA gene sequences by the neighbor-joining method with a bootstrap test of 1000 replicates was performed using MEGA 11 (<https://www.megasoftware.net>).

2.4. Synthesis of Indole-3-Acetic Acid

The production of indole-3-acetic acid was estimated by colorimetric method using Salkowski's reagent (0.1 g $(\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O})$ per 100 mL 50% H_2SO_4). The strains were cultured in a medium (g/L): glucose—10, NaCl—0.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.1, KCl—0.2, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ —0.002, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —0.002, yeast extract—0.5, KNO_3 —0.4, KH_2PO_4 —2.5, K_2HPO_4 —5 for 48 h. Tryptophan was not added. For analysis, 1.8 mL of Salkowski's reagent was added to 0.9 mL of the supernatant. The mixture was left for 30–60 min. A standard calibration curve was constructed using commercial indole-3-acetic acid (Fluka Biochemika, Darmstadt, Germany) in the same medium at concentrations ranging from 0 to 100 $\mu\text{g/mL}$. Measurements were made on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 530 nm.

2.5. Determination of Siderophore Synthesis

The ability of the studied strains to secrete siderophores was determined in vitro using the standard Chrome Azural S (CAS) method [16]. Bacterial cultures (previously cultivated on iron-free medium) were inoculated onto the surface of the solidified medium by forming a macrocolony using an inoculating loop. The culture plates were incubated for 2–5 days at 28 °C. The result was assessed by the change in the color of the agarized medium around the bacterial colonies from blue to orange, which indicates the presence of siderophores secretion.

2.6. Phosphate Solubilization Capacity

Phosphate solubilization capacity was assessed quantitatively using a colorimetric method. Strain cultures were grown on a medium containing (g/L): glucose—10, $\text{Ca}_3(\text{PO}_4)_2$ —5, $(\text{NH}_4)_2\text{SO}_4$ —0.5, NaCl—0.2, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ —0.1, KCl—0.2, $\text{MnSO}_4 \times 7\text{H}_2\text{O}$ —0.002, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ —0.002, yeast extract—0.5. The bacteria were cultured for 48 h, and the cell suspension was centrifuged (Eppendorf, Hamburg, Germany.). For analysis, 2.5 mL of 5% ammonium molybdate solution and 2.5 mL of hydrazine sulfate solution (120 mg/250 mL) were added to 0.9 mL of the supernatant, and the solution was made up to 25 mL with distilled water. The mixture was then heated in a water bath for 10 min at 90 °C. Optical density was measured using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength of 830 nm. A standard calibration curve was constructed based on a commercial $\text{Ca}_3(\text{PO}_4)_2$ (Fluka Biochemika, Darmstadt, Germany) in the same medium with concentrations from 0 to 3 mg/mL. Supernatant measurements were performed in triplicate on a Shimadzu UV-1800 spectrophotometer at a wavelength of 830 nm.

2.7. Antimicrobial Activity Assay

In order to evaluate the antimicrobial activity of isolated bacterial strains, the formation of the inhibition zone of test cultures was also studied. For this study, new isolates were pre-grown in liquid LB medium and the bacterial suspension in the exponential growth phase was directly taken to study the antagonistic activity. 100 µL of test culture in the exponential growth stage were applied to Petri dishes with solid LB medium and evenly distributed with a spatula over the entire surface of the dish. Sterile filter paper discs were placed on top of the test culture and 10 µL of bacterial suspension was applied. The results were evaluated after 24 h of cultivation at 25 °C by the presence of a lysis zone around the paper disc. The presence of this zone indicates the activity of the new isolates against the test culture.

2.8. Assay of the Effect of Novel Isolates on Seed Germination

Seeds of two wheat varieties, designated Tulaykovskaya 10 (Russia) and Darkhan 212 (Mongolia), were used in the study. All wheat seeds were pre-sterilized in 4% sodium hypochlorite and then washed three times with sterile distilled water. These seeds were then used in the experiments. In each experimental group, 50 seeds of each variety were used. Throughout the experiment, a control group of sterile plants that had not been treated in any way (except with sterile water, to standardize the amount of liquid applied to each plant) was maintained, which served as proof of the sterility of the initial treatment.

2.8.1. Phytostimulating Activity Assay

Sterile wheat seeds of two varieties, Tulaykovskaya 10 (Russia) and Darkhan 212 (Mongolia), were soaked in the culture broth of the studied strains. The seeds were treated with the culture broth for 12 h at 28 °C with constant stirring. The seeds were then placed on 0.8% starvation agar and allowed to germinate for 2–3 days at 20 °C. The resulting seedlings were evaluated for the percentage of seed germination, the length of the green biomass and roots, and the total biomass after germination. Total biomass was determined by the sum of the green and root masses. Mass was determined gravimetrically. Weighing was performed after drying the green biomass and roots at 90 °C until constant mass was reached.

2.8.2. Protective Function Assay

The following experimental and control groups were established: (1) sterile wheat seeds were treated with sterile water for 12 h and placed on 0.8% starvation agar, (2) wheat seeds were treated with the culture liquid of the studied strains for 12 h and placed on 0.8% starvation agar, (3) wheat seeds were treated with a mixture of the culture liquid of the studied strains and a spore preparation of the strain *Fusarium culmorum* VKM F-2303 in equal proportions for 12 h and placed on 0.8% agar, (4) wheat seeds were treated with the culture liquid of the studied strains for 12 h and placed on 0.8% agar, and then a spore suspension of *Fusarium culmorum* VKM F-2303 was applied.

2.9. Assay of the Effect of Novel Isolates on Plant Growth

40 sterile wheat seeds were treated with a bacterial suspension for 12 h at 28 °C with constant stirring and 40 sterile wheat seeds were treated with sterile water with the same conditions. The seeds were then placed on the surface of 0.8% starvation agar to germinate for 2–3 days at 28 °C. Then the seeds were transferred to a test tube with MS medium 4.3 g/L (Duchefa Biochemie, Haarlem, The Netherlands) and placed in a climate chamber (Russia) at the 20 °C temperature, 70–80% humidity, and light conditions 16/8 h day/night. After 2 days of cultivation, half of the plants (20 pretreated with bacterial suspension and 20 pretreated with sterile water) were treated a second time with the bacterial suspension. After 5 days of cultivation, half of the plants in all experimental groups were treated with a spore suspension of *Fusarium culmorum* VKM F-2303. The experimental procedure can be found in the Supplementary Materials (Figure S1). Controls included a negative control (sterile untreated seeds) and a positive control (sterile seeds treated with fungal spores). All procedures were performed in triplicate for each experimental group.

The measurement of the length of the green part and roots, as well as the determination of the total biomass after germination, were carried out in the same way as described above.

2.10. Microscopy Assay

2.10.1. Phase Contrast Microscopy

Microscopic studies were conducted using a Nikon Eclipse Ci microscope (Nikon, Tokyo, Japan) with a ProgRes SpeedXT camera (Jenoptic, Jena-Lobeda, Germany).

2.10.2. Fluorescence Microscopy

Plants were tested for the stability of their association with the tested bacteria and the localization of microbial cells in the rhizosphere. An Axiocam 506C camera (ZEISS, Jena, Germany) was used for photography. The roots of a plant colonized with bacteria were removed from the nutrient medium with sterile tweezers, then placed on a glass slide, 10 µL of buffer was added, and the slide was covered with a coverslip. To stabilize the cell cultures during the experiment, 50 mM HEPES-KOH buffer (catalog no. 11344041, ThermoFisher, Waltham, USA) + 330 mM sorbitol, pH 7.2, was used. SYTO 9 Green Fluorescent Nucleic Acid Stain (catalog no. S34854, ThermoFisher, USA) was used to stain bacterial nucleic acids. Next, the green signal from SYTO 9 was detected in bacteria in fluorescence mode and the lower lamp was additionally turned on to obtain images in dual mode: bright-field for visualization of plant tissue and fluorescence for visualization of bacteria. Plant tissues have a red tint, since the light path of the objective lens contains a 650 nm double-cutoff filter. Fluorescence was detected using a 56HE filter set (Carl Zeiss MicroImaging GmbH, Hallbergmoos, Germany) at excitation and emission wavelengths of 490 nm and 512 + 630 nm, respectively. Microscopic

examination of plants colonized by the strains was performed using a Zeiss Axio Imager A1 fluorescence microscope (Zeiss, Jena, Germany) equipped with an Axiocam 506 camera (Carl Zeiss MicroImaging GmbH, Hallbergmoos, Germany).

2.10.3. Scanning Microscopy

The root surface morphology of colonized plants was examined using scanning electron microscopy (SEM). Root samples placed on membrane filters were fixed at 4 °C for 24 h in glutaraldehyde vapor and then postfixed at 20 °C for 3 h in OsO₄ vapor. After dehydration in propylene oxide vapor, the samples were sputter-coated with gold (Fine Coat Ion Sputter JFC-1100, Tokyo, Japan) and examined using a JSM-6510LV scanning microscope (JEOL, Tokyo, Japan).

2.11. Antibiotic Susceptibility Assessment

The disc diffusion method was utilized to evaluate the level of sensitivity or resistance of those selected bacterial isolates. A set of 15 antibiotic disks (CJSC NICEF, St. Petersburg, Russia) was used in accordance with the manufacturer's instructions. The cultured bacterial strains (48 h at 28 °C) were inoculated and evenly distributed on plate containing LB growth medium. Then antibiotic disks were applied on the surface of the plate. The presence of a clear zone (zone of inhibition) after incubation period (24–48 h at 28 °C) was considered the criterion for antibiotic susceptibility.

2.12. Photosynthetic Activity in Wheat Leaves

The functional status of the photosynthetic apparatus in wheat leaves was studied using a MULTI-COLOR PAM fluorometer (Waltz, Germany), which determines photosystem II activity based on chlorophyll fluorescence. All plants were dark-adapted for at least 30 min before measurements, which were performed on the middle part of the second leaf from the top of each plant. Photosynthetic parameters of photosystem II were calculated according to equations provided in the instrument manual. The maximum quantum yield of photosystem II was determined as F_v/F_m , where $F_v = F_m - F_o$, F_o is the initial fluorescence, and F_m is the maximum chlorophyll fluorescence obtained with a saturating 500 ms flash ($\lambda = 625$ nm, $\sim 12,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in dark-adapted plants. The effective quantum yield of photosystem II ($Y(\text{II})$) was calculated as $Y(\text{II}) = (F_m' - F_s)/F_m'$, where F_m' is the flash-induced maximum chlorophyll fluorescence and F_s is the stationary fluorescence level in light-adapted plants ($\lambda = 625$ nm, $\sim 200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

2.13. Statistical Analysis

All studies were conducted in at least triplicate. All statistical procedures were performed using SPSS version 23.0 (IBM Corporation, New York, NY, USA) and Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). Differences were tested using a *t*-test for independent samples or one-way analysis of variance (ANOVA). If ANOVA indicated significant differences, post hoc multiple comparisons were performed using Tukey's Honest Significant Difference (HSD) test. The level of significance was set at $p \leq 0.05$. All data are presented as the mean $\pm$ standard deviation (SD). Different superscript letters (a, b, c, etc.) in the tables indicate statistically significant differences between treatment groups.

3. Results

3.1. Isolation and Selection of Isolates

From the upper green layer of *Aegilops cylindrica*, seven pure culture strains were isolated: WP1, WP3, WP4, WS6, WF1, WF2, and WF3. The following characteristics were assessed in the isolates: synthesis of auxin and siderophores, phosphate solubility, antimicrobial activity, and seed germination stimulation.

All three PGP properties varied significantly among the isolates ($p < 0.001$). All isolates demonstrated the ability to synthesize auxins (Table 1), with concentrations ranging from 1.56 ± 0.11 $\mu\text{g/mL}$ (for WP3) to 5.09 ± 0.08 $\mu\text{g/mL}$ (for WF1). The ability to synthesize siderophores was assessed by the formation of a clear zone on an agar medium (Table 1). The new strains can synthesize siderophores, with strain WP3 exhibiting the highest activity; the clear zone reached 26 mm after 5 days of cultivation (Figure 1).

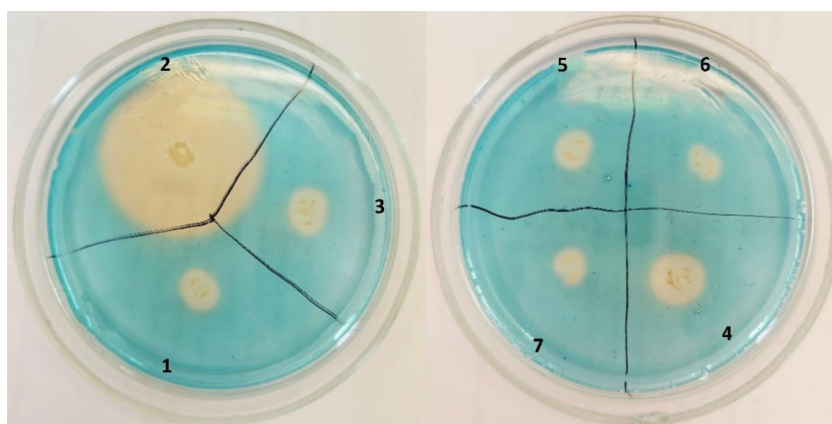


Figure 1. Evaluation of siderophore synthesis by new isolates: 1—WP1, 2—WP3, 3—WP4, 4—WS6, 5—WF1, 6—WF2, 7—WF3.

Table 1. PGP-properties of new isolates.

Strain	Auxin, $\mu\text{g/mL}$	Synthesis of Siderophores, mm	Phosphate Concentration, $\mu\text{g/mL}$
WP1	$4.09^d \pm 0.09$	$3^c \pm 0.12$	$7.26^b \pm 0.21$
WP3	$1.56^f \pm 0.11$	$26^a \pm 0.96$	$1.49^f \pm 0.05$
WP4	$4.40^{b,c} \pm 0.20$	$4^c \pm 0.21$	$4.47^d \pm 0.09$
WS6	$4.78^{a,b} \pm 0.19$	$7^b \pm 0.20$	$4.03^e \pm 0.05$
WF1	$5.09^a \pm 0.08$	$4^c \pm 0.12$	$5.00^c \pm 0.12$
WF2	$3.35^e \pm 0.12$	$3^c \pm 0.22$	$12.05^a \pm 0.28$
WF3	$4.33^{c,d} \pm 0.20$	$3^c \pm 0.09$	$7.07^b \pm 0.20$

Different letters (a–f) within each column indicate statistically significant differences between strains ($p < 0.05$).

Analysis of the isolates' phosphate solubilization activity revealed that strain WF2 exhibited the highest efficiency in dissolving inorganic phosphate (Table 1). Comparative analysis revealed that all isolates possessed PGP capabilities with varying levels of effectiveness.

3.2. Antimicrobial Activity of Isolates

The ability to inhibit bacteria and filamentous fungi was evaluated using 18 bacterial strains and 8 fungal strains (Table 2). Isolate WS6 demonstrated the greatest effect in terms of both potency and the range of inhibited strains: it inhibited the growth of 14

bacterial strains and 7 fungal strains, including *Fusarium culmorum* VKM F-2303 (Figure 2). Isolates WP1, WP4, and WF2 demonstrated a moderate level of inhibition of the tested strains, including phytopathogenic fungi.

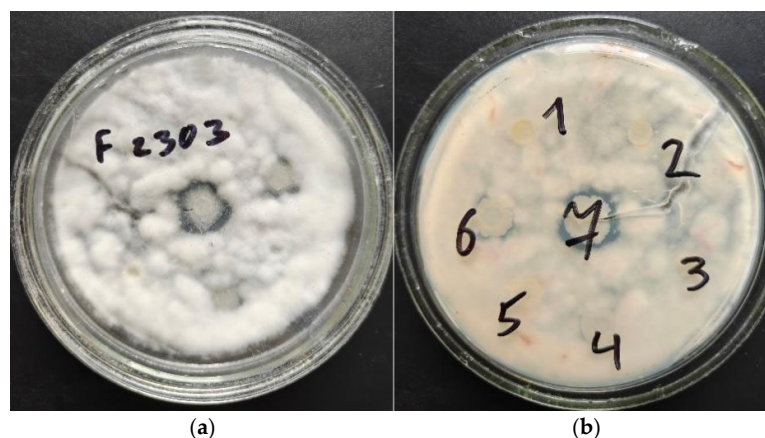


Figure 2. The ability of new isolates to inhibit the growth of the grain phytopathogen *Fusarium culmorum* VKM F-2303: 1—WF1, 2—WF2, 3—WF3, 4—WP1, 5—WP3, 6—WP4, 7—WS6. (a) top of the Petri dish (aerial mycelium, visible part of the fungus); (b) bottom of the Petri dish (zones of growth inhibition are visible).

Table 2. Antimicrobial activity of new isolates.

No.	Microorganisms	WP1	WP3	WP4	WS6	WF1	WF2	WF3
Bacteria								
1	<i>Aeromonas veronii</i> LS1	$1^c \pm 0.08$	—	—	$1^c \pm 0.5$	$1^c \pm 0.08$	$1^c \pm 0.07$	$2^c \pm 0.5$
2	<i>Alcaligenes faecalis</i> VKM B-1518	—	—	—	$2^b \pm 0.5$	$2^b \pm 0.5$	$6^a \pm 0.8$	$3^b \pm 0.6$
3	<i>Arthrobacter</i> sp. B52	—	—	—	$4^b \pm 0.9$	—	$8^a \pm 0.9$	$4^b \pm 0.5$
4	<i>Bacillus mucoides</i> KBA4	$0.5^c \pm 0.05$	—	—	$1^c \pm 0.08$	—	$0.5^c \pm 0.1$	$2^c \pm 0.5$
5	<i>Bacillus subtilis</i> ATCC 6633	$2^c \pm 0.5$	—	$1.5^c \pm 0.2$	$5^a \pm 0.7$	$2^b \pm 0.5$	$2^b \pm 0.8$	$3^b \pm 0.5$
6	<i>Curtobacterium flaccumfaciens</i> Ac-2884	$3^b \pm 0.5$	—	$4^b \pm 0.5$	$1^c \pm 0.05$	—	—	—
7	<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i> CFBP 3418	$1^c \pm 0.07$	$2^b \pm 0.4$	$2^b \pm 0.5$	$3^b \pm 0.75$	$1^c \pm 0.08$	—	—
8	<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i> VKM Ac-1923	—	$3^b \pm 0.5$	$3^b \pm 0.4$	$3^b \pm 0.4$	—	—	—
9	<i>Escherichia coli</i> C600	—	—	—	—	$3^b \pm 0.9$	$5^a \pm 0.5$	$7^a \pm 0.5$
10	<i>Escherichia coli</i> S	—	$3^b \pm 0.5$	—	$9^a \pm 0.6$	$4^b \pm 0.5$	$15^a \pm 2.1$	$9^a \pm 1.5$
11	<i>Kocuria rosea</i> VKM B-1236	$3^b \pm 0.5$	—	—	—	$6^a \pm 0.8$	$7^a \pm 1.2$	$4^b \pm 0.8$
12	<i>Pantoea agglomerans</i> ATCC 27155	—	—	—	$1^c \pm 0.4$	—	—	—
13	<i>Pectobacterium carotovorum</i> B15	—	—	—	—	—	—	—
14	<i>Pectobacterium carotovorum</i> VKM B-1247	—	$2^b \pm 0.4$	—	$2^b \pm 0.5$	—	$1^c \pm 0.08$	—
15	<i>Pseudomonas aeruginosa</i> ML	—	—	—	$4^b \pm 0.5$	—	—	—
16	<i>Pseudomonas protegens</i> 38a	—	—	$2^b \pm 0.4$	$1^c \pm 0.09$	$7^a \pm 0.7$	$7^a \pm 0.5$	$7^a \pm 0.8$
17	<i>Pseudomonas syringae</i> VKM B-1546	—	—	—	—	$2^b \pm 0.6$	—	$3^b \pm 0.6$
18	<i>Staphylococcus</i> sp. St35	—	—	$1.5^c \pm 0.2$	$4^b \pm 0.5$	—	$3^b \pm 0.5$	$5^a \pm 0.6$
Mycelial fungi								
1	<i>Alternaria brassicicola</i> VKM F-1864	2 ± 0.5	—	—	$15^a \pm 1.5$	—	$2^b \pm 0.5$	—
2	<i>Bipolaris sorokiniana</i> VKM F-4006	$3^b \pm 0.6$	—	$1^c \pm 0.07$	$6^a \pm 0.12$	—	$2^b \pm 0.4$	—
3	<i>Botrytis cinerea</i> VKM F-4549	$2^b \pm 0.5$	$1^c \pm 0.04$	$2^b \pm 0.5$	$3^b \pm 0.5$	—	—	—
4	<i>Fusarium avenaceum</i> VKM F-132	$1^c \pm 0.06$	—	$2^b \pm 0.4$	$2^b \pm 0.5$	—	$5^a \pm 0.4$	—
5	<i>Fusarium culmorum</i> VKM F-2303	—	—	$3^b \pm 0.5$	$4^b \pm 0.5$	$2^b \pm 0.8$	—	$2^b \pm 0.3$

6	<i>Penicillium gladioli</i> VKM F-2088	$1^c \pm 0.09$	–	–	–	–	$2^b \pm 0.5$	–
7	<i>Pythium ultimum</i> VKM F-4782	$3^b \pm 0.5$	–	$1^c \pm 0.08$	$3^b \pm 0.5$	$1^c \pm 0.08$	$1.5^c \pm 0.1$	$2^b \pm 0.5$
8	<i>Pythium vexans</i> VKM F-1193	–	–	–	$2^b \pm 0.5$	–	$6^a \pm 0.8$	$3^b \pm 0.5$

Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$). Inhibition zones were classified as: strong (>6 mm), moderate (2–6 mm), or weak (<2 mm). “–” no inhibition zone detected.

3.3. Effect of New Isolates on Seed Germination

A culture medium consisting of a suspension of the strains under study was used to soak seeds of the Tulaykovskaya 10 wheat variety. It was found that strains WF1, WF2, and WF3 had no positive effect on the germination of wheat seeds of any variety, and when high cell concentrations were used in the suspension, they completely inhibited germination. The remaining four isolates had a noticeable positive effect on the germination rate of Tulaykovskaya 10 wheat: the control group had a germination rate of 44.4%, while after treatment with strains WP1, WP3, WP4, and WS6, the rates were 62.2%, 60%, 77.8%, and 77.8%, respectively (Figure 3). The total biomass of germinated seeds (total roots and aboveground parts) was (mg per seed): 5.0, 7.5, 5.1, 8.4, and 7.4, respectively (Table 3).

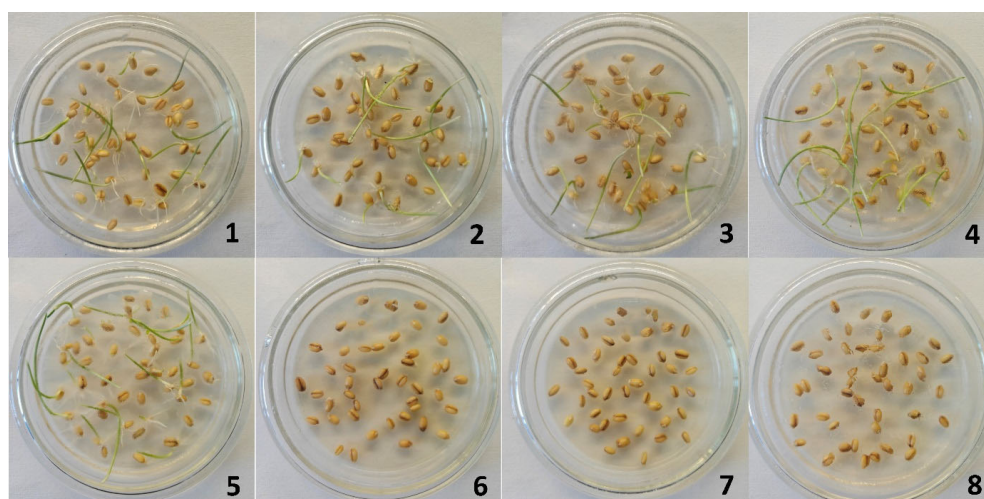


Figure 3. The effect of treatment on wheat seed germination: 1—control (without treatment), 2—WP1 treatment, 3—WP3 treatment, 4—WP4 treatment, 5—WS6 treatment, 6—WF1 treatment, 7—WF2 treatment, 8—WF3 treatment.

Table 3. Effect of new isolates on seed germination.

Control		WP1		WP3		WP4		WS6	
Roots, mg/seed	Stems, mg/seed	Roots, mg/seed	Stems, mg/seed	Roots, mg/seed	Stems, mg/seed	Roots, mg/seed	Stems, mg/seed	Roots, mg/seed	Stems, mg/seed
$0.5^c \pm 0.05$	$4.5^a \pm 0.7$	$2.5^a \pm 0.4$	$5.0^a \pm 0.5$	$1.2^{b,c} \pm 0.2$	$3.9^a \pm 0.7$	$2.7^a \pm 0.6$	$5.7^a \pm 0.9$	$2.5^a \pm 0.7$	$4.9^a \pm 0.6$
Total biomass: 5.0^c		Total biomass: 7.5^{ab}		Total biomass: 5.1^c		Total biomass: 8.4^a		Total biomass: 7.4^b	

Different letters (a, b, c) indicate statistically significant differences between treatment groups ($p < 0.05$). In the seed germination data, a one-way analysis of variance revealed significant differences in root mass ($p < 0.05$) and total biomass ($p < 0.05$), but not in stem mass ($p > 0.05$).

The germinated seeds were transferred to MS medium and cultured for 14 days, after which the biomass growth (above-ground and root) of the wheat plants was assessed (Figure 4).



Figure 4. Evaluation of the effect of treating seeds with a bacterial suspension of new isolates: 1—control (untreated), 2—WP1 treatment, 3—WP3 treatment, 4—WP4 treatment, 5—WS6 treatment.

No significant differences were found between the control (no treatment) and the experimental variants (treatment with strains WP1, WP3, WP4, and WS6) (Table 4). However, WS6 showed a higher stem length than WP3 and WP4 ($p < 0.05$), indicating a moderate growth-promoting effect, although it did not exceed the effect of the untreated control.

Table 4. Effect of new isolates on the plant growth.

Variants	Part of the Plant	Average Length, mm	Weight, mg per 1 Plant	Total Biomass, mg per 1 Plant
Control	Roots	67.7 ^a ± 10.3	4.1 ^a ± 0.5	36 ^a
	Stems	221.7 ^{a,b} ± 24.1	31.9 ^a ± 3.2	
WP1	Roots	76.4 ^a ± 12.4	4.1 ^a ± 0.8	37.1 ^a
	Stems	228.6 ^{a,b} ± 21.8	33.0 ^a ± 3.5	
WP3	Roots	70 ^a ± 11.0	5.1 ^a ± 0.6	30.4 ^a
	Stems	187.1 ^b ± 22.1	25.3 ^a ± 1.2	
WP4	Roots	75.4 ^a ± 14.0	5.0 ^a ± 0.5	36.7 ^a
	Stems	188 ^b ± 21.4	31.7 ^a ± 5.1	
WS6	Roots	63.1 ^a ± 10.9	5.3 ^a ± 0.8	39.6 ^a
	Stems	263.5 ^a ± 23.1	34.3 ^a ± 10.2	

Different letters (a, b) within the stem length indicate statistically significant differences between treatments ($p < 0.05$). For all other parameters (root length, root weight, stem weight or total biomass) no significant differences were found, and all treatments share the same letter (a).

Thus, strains WP4 and WS6 were selected for further work, as they are capable of effectively stimulating seed germination, have pronounced antimicrobial activity, in particular against the phytopathogenic fungus *Fusarium culmorum* VKM F-2303, and also have positive tests for potential PGP activity.

Identification by 16S rRNA gene nucleotide sequencing revealed that all isolated strains belong to the genus *Bacillus* (Figure S2). Closest relatives were identified for strains WP4 and WS6: *Bacillus mojavensis* WP4 and *Bacillus siamensis* WS6 (Figure 5).

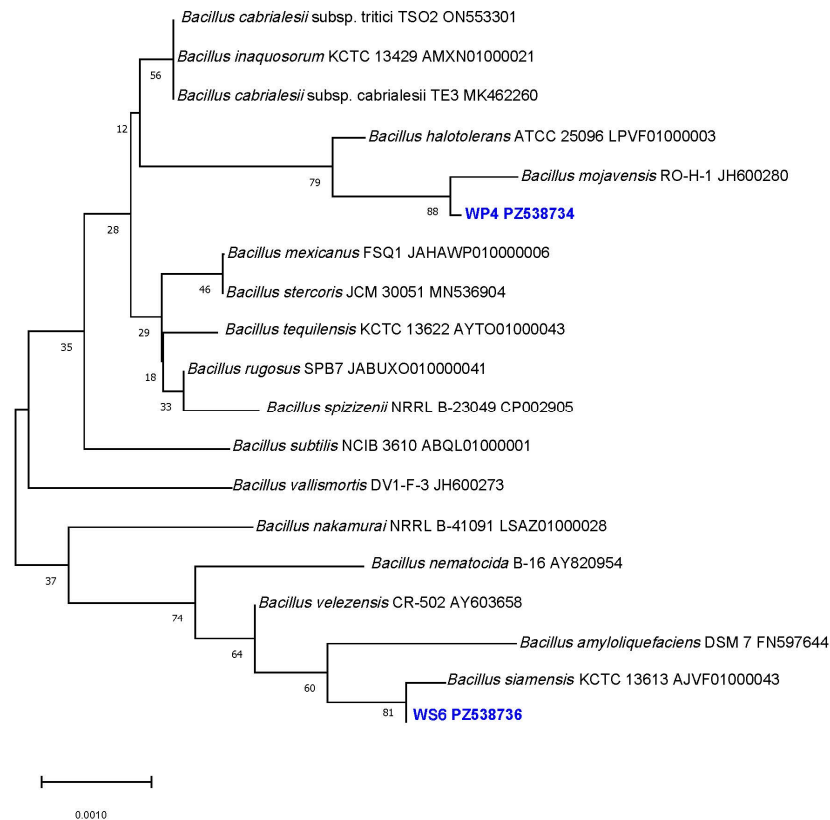


Figure 5. Phylogenetic tree constructed based on 16S rRNA nucleotide sequences for strains WP4 and WS6.

3.4. Colonization of Roots by Bacteria

Microscopic analysis of the colonized plants Tulaykovskaya 10 and Darkhan 212 revealed the presence of microorganisms on the root surface, where bacterial cells had colonized the surface of the root hairs. Since the bacteria we used occur naturally mainly in the rhizosphere of plants, their highest concentration is observed in the roots (Figures 6 and 7). However, the rhizosphere is not a unique niche for these species, so they are also found in the phyllosphere.

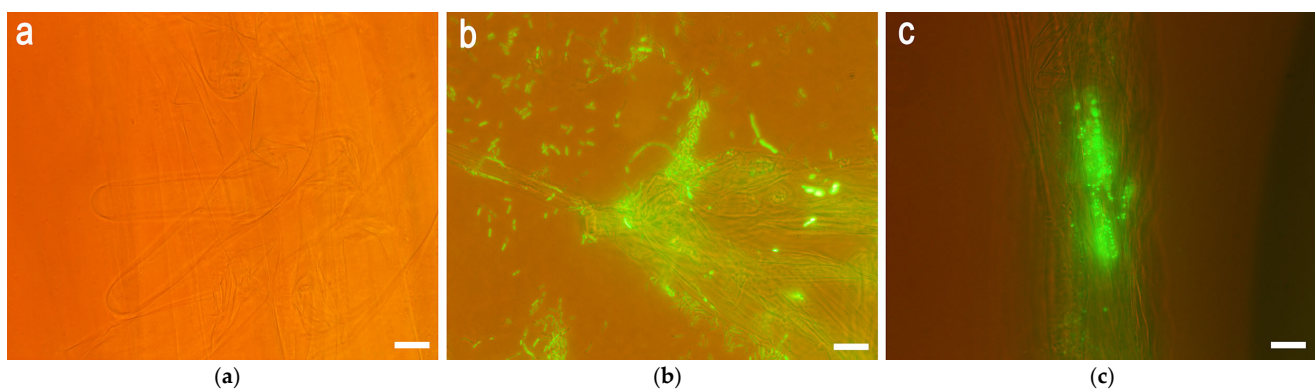


Figure 6. Microscopic analysis of colonized Tulaykovskaya 10 wheat plants grown in vitro. (a) The root hair of the control plant; (b) The root hair with the bacterial strain WP4, (c) The root hair with the bacterial strain WS6. The age of the plants is 5 days. The scale segment is 5 microns.

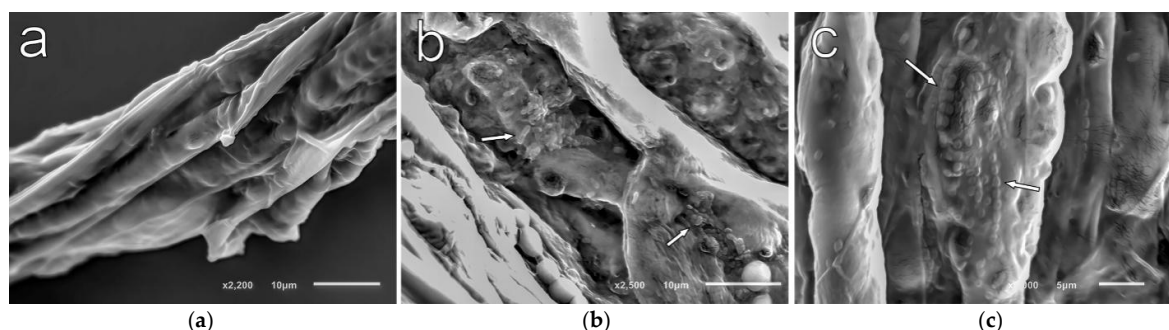


Figure 7. Scanning electron microscopy of colonized Darkhan 212 wheat plants grown in vitro. (a) The surface of the root of the control plant. (b) The surface of the root with the bacterial strain WS6. (c) The surface of the root with a bacterial strain WP4. The plants were approximately 3 weeks old. The arrows indicate bacteria

Thus, the obtained microscopic data provide direct visual evidence that the studied bacteria form an association with wheat roots.

3.5. Antibiotic Resistance Assessment

All isolates capable of stimulating wheat seeds were tested for resistance to a broad spectrum of antibiotics (Table 5). Antimicrobial susceptibility testing revealed that the strain WS6 demonstrated resistance to rifampicin (5 µg) and ceftazidime (30 µg), while strain WP1 demonstrated resistance to cefoperazone/sulbactam (75 µg).

Table 5. Antibiotic Resistance of new isolates.

No.	Antibiotic	Concentration, µg	WP1	WP3	WP4	WS6
1	Amoxicillin/Clavulanic acid	20	+	+	+	+
2	Ampicillin	10	+	+	+	+
3	Vancomycin	30	+	+	+	+
4	Gentamicin	120	+	+	+	+
5	Imipenem	10	+	+	+	+
6	Linezolid	30	+	+	+	+
7	Levofloxacin	5	+	+	+	+
8	Benzylpenicillin	10	+	+	+	+
9	Rifampicin	5	+	+	+	–
10	Ceftazidime	30	+	+	+	–
11	Cefazolin	30	+	+	+	+
12	Cefoxitin	30	+	+	+	+
13	Cefepime	30	+	+	+	+
14	Cefoperazone/Sulbactam	75	–	+	+	+
15	Erythromycin	15	+	+	+	+

“+” – indicates that the strain is sensitive to the antibiotic (the growth suppression zone is ≥8 mm);

“–” – indicates that the strain is resistance to the antibiotic (the growth suppression zone is ≤8 mm).

3.6. Effect of Isolates of *Bacillus mojavensis* WP4 and *Bacillus siamensis* WS6 on Seed Germination

All experimental seed groups were pre-soaked in a cell suspension of the selected strains. After 5 days of germination, it was shown that, although no significant effect on the germination rate was observed, a positive effect of the WP4 and WS6 bacterial suspensions was noted for the Tulaykovskaya 10 wheat variety. Treatment with WP4 resulted in a 2.2-fold increase in total biomass (the sum of root mass and above-ground

green biomass), while treatment with WS6 resulted in a 2.9-fold increase. For the Darkhan 212 wheat variety, no significant effect was observed following treatment with the cell suspensions of the tested isolates (Table 6).

Table 6. Effect of WP4 and WS6 isolates on seed germination.

Parameter	Control	WP4	WS6
Tulaykovskaya 10			
number of sprouted seeds, %	81.3 ^a ± 12.3	79.6 ^a ± 12.4	81.2 ^a ± 15.5
average length of green shoot, mm	42 ^b ± 6.7	63.6 ^a ± 10.3	67.8 ^a ± 12.1
average length of root, mm	31.7 ^a ± 5.6	41.6 ^a ± 4.2	46.7 ^a ± 12.5
green shoot, mg/seed	1.1 ^{a,b} ± 0.7	2.2 ^{a,b} ± 0.8	2.9 ^a ± 0.5
roots, mg/seed	0.3 ^b ± 0.09	1.0 ^a ± 0.07	1.2 ^a ± 0.4
weight, mg/seed	1.4 ^b ± 0.7	3.1 ^a ± 0.7	4.1 ^a ± 0.7
Darkhan 212			
number of sprouted seeds, %	93 ^a ± 14.8	96.5 ^a ± 14.3	93.8 ^a ± 13.9
average length of green shoot, mm	57.2 ^a ± 10.2	60.7 ^a ± 12.5	58.6 ^a ± 12.1
average length of root, mm	53.5 ^a ± 11.0	67.4 ^a ± 10.1	55.4 ^a ± 11.9
green shoot, mg/seed	3.4 ^a ± 0.8	3.5 ^a ± 0.7	3.3 ^a ± 0.4
roots, mg/seed	2.2 ^a ± 0.9	2.3 ^a ± 0.5	2 ^a ± 0.09
weight, mg/seed	5.6 ^a ± 0.7	5.8 ^a ± 0.6	5.2 ^a ± 0.8

Different letters (a, b) in each row (i.e., for each variety and strain separately) indicate statistically significant differences between treatment options ($p < 0.05$).

A 21-day observation of the experimental groups showed that treating young wheat plants with a bacterial suspension did not further stimulate their growth.

3.7. Evaluation of the Protective Capabilities of New Isolates

Close attention was paid to the effect of the protective properties of the isolates on seeds inoculated with *Fusarium culmorum* VKM F-2303 spores. It was found that in the positive control (no treatment with bacterial cells + *Fusarium culmorum* VKM F-2303), the percentage of germinated seeds for both wheat varieties was significantly reduced. In the treated groups, a protective effect of both strains was observed: the percentage of germinated seeds increased by 1.5–2 times compared to the control (Table 7).

Table 7. Protective properties of WP4 and WS6 isolates against *Fusarium culmorum* VKM F-2303 on wheat seeds.

No.	Experimental Group	Number of Sprouted Seeds, %
Tulaykovskaya 10		
1	Control Tulaykovskaya 10	57.9 ^{c,d} ± 5.2
2	Tulaykovskaya 10 + VKM F-2303	28.6 ^f ± 3.8
3	Tulaykovskaya 10 + WP4	78.4 ^{a,b} ± 6.1
4	Tulaykovskaya 10 + WS6	80.5 ^a ± 6.9
5	Tulaykovskaya 10 + (WP4 + VKM F-2303)	52.6 ^{c,d,e} ± 6.0
6	Tulaykovskaya 10 + (WS6 + VKM F-2303)	51.1 ^{d,e} ± 4.3
7	(Tulaykovskaya 10 + WP4) + VKM F-2303	52.3 ^{c,d,e} ± 5.6
8	(Tulaykovskaya 10 + WS6) + VKM F-2303	51.3 ^{d,e} ± 4.8
Darkhan 212		
1	Control Darkhan 212	77.4 ^{a,b} ± 5.6
2	Darkhan 212 + VKM F-2303	37.5 ^e ± 5.3
3	Darkhan 212 + WP4	93.5 ^a ± 7.8

4	Darkhan 212 + WS6	81.2 ^{a,b} ± 5.2
5	Darkhan 212 + (WP4 + VKM F-2303)	55.3 ^{b,c,d} ± 5.1
6	Darkhan 212 + (WS6 + VKM F-2303)	58.8 ^{b,c} ± 4.8
7	(Darkhan 212 + WP4) + VKM F-2303	51.6 ^d ± 4.9
8	(Darkhan 212 + WS6) + VKM F-2303	53.1 ^{c,d} ± 4.5

Different letters (a–f) within each column (i.e., for each variety separately) indicate statistically significant differences between treatments ($p < 0.05$).

No significant differences were observed when young plants were treated with *Fusarium culmorum* VKM F-2303. In the control treatments for both wheat varieties (which were not treated with bacterial strains), isolated cases of root rot were observed, whereas in the experimental treatments, all plants remained healthy.

3.8. Effect of Bacterial Treatment on Photosystem II Activity in Young Wheat Plants

Analysis of these chlorophyll fluorescence parameters showed that infection of wheat with the fungus *Fusarium culmorum* VKM F-2303 caused pronounced suppression of photosynthetic activity of PSII in both studied varieties, Tulaykovskaya 10 and Darkhan 212, while the most severe reduction was observed for the Y(II) parameter (Figure 8).

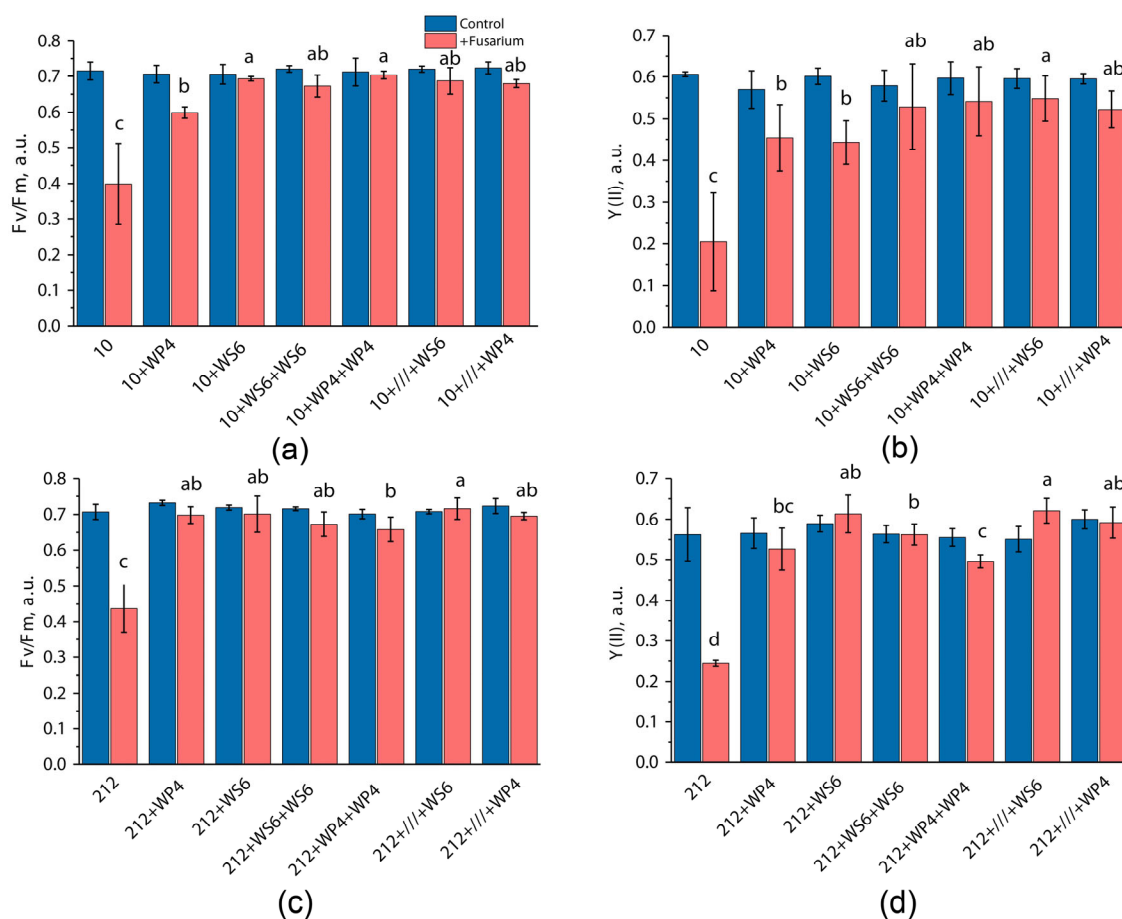


Figure 8. Effect of WP4 and WS6 isolates treatment on Photosystem II activity in young wheat plants. (a,b) Tulaykovskaya 10; (c,d) Darkhan 212. Different letters (a, b, c, d) within each column indicate statistically significant differences between treatments ($p < 0.001$).

In the Tulaykovskaya 10 variety, which was treated with the pathogen alone, the Fv/Fm ratio dropped sharply to approximately 0.40 compared to the control (~0.72), while the Y(II) index decreased by more than a factor of three, from ~0.60 in the control to ~0.20. This indicates a significant disruption in the functional stability of the photosynthetic apparatus under the influence of *Fusarium culmorum* VKM F-2303 infection. Pretreatment of the plants with strains WP4 and WS6 helped preserve PSII activity and significantly reduce the negative impact of the pathogen.

The most pronounced protective effect in variety Tulaykovskaya 10 was observed with isolate WS6. After primary seed treatment with WS6, the Fv/Fm values in infected plants detected close to control levels (~0.69–0.70), while Y(II) was lower only by ~25% (~0.44–0.53). Treatment with isolate WP4 also improved the physiological state of the photosynthetic apparatus in plants, although the effect was somewhat less pronounced in the case of Fv/Fm. Under double treatment (seed soaking followed by subsequent application to young plants), Fv/Fm and Y(II) parameters remained at a consistently high level, indicating a prolonged protective effect of bacterial inoculation.

In variety Darkhan 212, the negative effect of the pathogen was also accompanied by a decrease in photochemical activity of PSII in leaves; however, the degree of the suppression was less severe compared to Tulaykovskaya 10. In the pathogen-only variant, Fv/Fm decreased to approximately 0.44, and Y(II) to ~0.31. At the same time, WP4 and WS6 bacteria completely preserved PSII functional stability.

In the absence of a pathogen (Control, Figure 8), none of the bacterial treatments had a significant effect on Fv/Fm or Y(II) in any of the varieties compared to the untreated control ($p > 0.05$). This suggests that bacterial strains do not inherently alter the efficiency of photosystem II but act specifically under conditions of pathogen stress.

4. Discussion

In this study, two strains of the epiphytic bacteria—*Bacillus mojavensis* WP4 and *Bacillus siamensis* WS6—were isolated and characterized from the phyllosphere of the wild cereal *Aegilops cylindrica*.

The results demonstrate that these strains possess a complex of properties characteristic of PGP-bacteria, including the synthesis of auxins, siderophores, and phosphate-solubilizing activity, and also exhibit antagonism toward the phytopathogenic fungus *Fusarium culmorum*.

Using wild relatives of cultivated plants as a source for isolating PGP bacteria is a promising strategy. Over the course of evolution, wild grasses growing in natural ecosystems have developed effective mechanisms for interacting with microorganisms in the rhizosphere and phyllosphere, making them a reservoir of “beneficial” bacteria with enhanced PGP properties and antagonistic activity [17]. The strains we obtained belong to the species *B. mojavensis* and *B. siamensis*, which are widely known to produce secondary metabolites with antimicrobial activity and are often considered biocontrol agents [18,19].

According to the literature, *B. mojavensis* can produce volatile organic compounds (VOCs), which act as plant growth modulators [20]. *B. mojavensis* has also been reported to reduce mycotoxin accumulation in corn [21]. In addition to its activity, *B. mojavensis* can also produce cyclic lipopeptides (CLPs), which include the iturin, surfactin, fengycin family [22,23].

B. siamensis is also considered an active member of the PGP group of bacteria [24]. It has been shown that members of this species also act as phosphate-solubilizing bacteria. In addition, *B. siamensis* colonizes plant tissues as an endophyte, promoting a symbiotic relationship that improves nutrient uptake and reduces levels of reactive oxygen species (ROS) [25]. An important characteristic of the selected strains is their ability to synthesize

siderophores and solubilize phosphates. These mechanisms increase the availability of iron and phosphorus to plants, which is particularly important under conditions of soil deficiency in these elements [26]. In our experiments, the *B. siamensis* WS6 strain demonstrated high activity in siderophore synthesis, while *B. mojavensis* WP4 demonstrated high phosphate solubilization activity.

The ability of the isolated strains to synthesize indole-3-acetic acid (IAA) is an important direct mechanism for stimulating plant growth. Our data on auxin production (from 1.56 to 5.09 µg/mL) are consistent with the results of other studies in which *Bacillus* spp. strains isolated from various sources produced IAA in similar or wider concentration ranges, effectively stimulating root growth [27]. The correlation between IAA synthesis and increased root and shoot biomass in wheat observed in our study confirms the key role of this phytohormone in the PGP effect.

The most significant result of this study is the demonstrated ability of strains WP4 and WS6 to inhibit the growth of *Fusarium culmorum* in vitro and to exert a protective effect during the germination stage of wheat seeds. *Fusarium culmorum* is one of the most destructive pathogens of wheat, causing root and basal rot, as well as fusarium head blight [2,3]. The observed antifungal effect is likely due to the production of a wide range of secondary metabolites by the isolated *Bacillus* spp. strains, including lipopeptides (surfactin, iturin, and fengycin), polyketides, and hydrolytic enzymes. Lipopeptides of the iturin and fengycin families are known to have potent antifungal activity, disrupting the integrity of hyphal membranes and inhibiting spore germination [22–25]. *B. siamensis* WS6, which demonstrated the broadest spectrum of antagonistic activity against phytopathogens (including *Alternaria*, *Bipolaris*, and *Fusarium* spp.) in our study, is, in our opinion, the most promising candidate for further study. Our data are consistent with the results of other research groups, which also report the high efficacy of *B. siamensis* in the biocontrol of *Fusarium culmorum* on wheat. Saadaoui et al. [30] showed that epiphytic bacteria, including *Bacillus mojavensis*, not only inhibit the growth of *F. culmorum* in vitro but also promote the accumulation of wheat plant biomass. Experiments on the effect of bacterial colonization of wheat seeds on their germination demonstrated a clear stimulatory effect of the WP4 and WS6 strains on the Tulaykovskaya 10 wheat variety, resulting in an increased germination rate and, most importantly, a significant increase in total seedling biomass (up to 2.9 times compared to the control). However, the effect on the Darkhan 212 variety was less pronounced, indicating cultivar-specific interactions between the bacteria and the host plant.

Despite the successful protection of seedlings against *F. culmorum* on agar plates, no significant protective effect of the bacterial treatment was observed when adult plants were infected (in experiments using MS medium). This may be due to several reasons: (1) experimental conditions (a sterile MS medium with limited carbon content in the nutrient solution could not fully replicate the rhizosphere interactions necessary for effective colonization and antagonism), (2) an insufficient interval between bacterial treatment and infection (two treatments may not be enough to maintain a large number of bacteria on the root surface), (3) the fact that the main defense mechanisms of *Bacillus* spp. against fusarium wilt may be more effective in the early stages of infection—during seed germination and seedling development [27]. Furthermore, it is known that *F. culmorum* penetrates the plant through root hairs and wound surfaces, and protection against the pathogen at this stage requires dense colonization of the soil by antagonistic bacteria.

One of the key and, in our opinion, most significant results of this study is the observed change in the protective effect of bacterial strains as plants transition from seedling to adult plant stage. While bacterial inoculation resulted in a significant reduction in the disease severity index during the early stages of vegetation, *Fusarium culmorum* caused comparable damage in both treated and control plants in adult plants.

This phenomenon may have several interrelated explanations. First, as plants age, root exudates undergo quantitative and qualitative changes: the content of easily digestible sugars and organic acids decreases, while the proportion of phenolic compounds and lignins increases [28]. This may lead to a decrease in the available nutritional sources for antagonist bacteria and, consequently, a decrease in their metabolic activity. Second, compaction and lignification of root tissue in mature plants reduces vulnerability to pathogen invasion, but can also create physical barriers that limit deep bacterial colonization and their penetration into active root growth zones [29]. Third, it cannot be ruled out that during late stages of the growing season, the expression of genes responsible for the production of antimicrobial metabolites is weakened, which requires separate study using molecular monitoring methods (e.g., qPCR to assess the expression of key biosynthetic genes).

Based on these observations, we believe that to achieve a sustained protective effect throughout the entire growing season, it is necessary to develop additional strategies aimed at increasing bacterial resilience and maintaining high metabolic activity in the rhizosphere during the later stages of development. In particular, promising approaches may include: 1) repeated inoculations at key stages of plant development (e.g., tillering and heading); 2) the use of protective carriers or microcapsules that ensure the gradual release of bacteria and nutrient substrates; 3) the creation of synthetic microbial consortia in which different strains perform complementary functions at various stages of the host plant's development. These issues will be the focus of our future research, aimed at overcoming the age-related decline in the effectiveness of biological control.

Although bacterial treatment did not prevent infection by the phytopathogen, it restored Photosystem II (PSII) activity in infected plants. Photosystem II (PSII) is considered one of the most vulnerable components of the photosynthetic apparatus of the thylakoid membrane. At the same time, the measurement of variable chlorophyll fluorescence makes it possible to rapidly and non-invasively assess both the maximum functional potential of PSII in the studied plants using the so-called parameter—the maximum quantum yield of PSII (F_v/F_m), and its activity under continuous illumination, characterized by the parameter—the effective quantum yield of PSII ($Y(II)$). Both indicators reflect the fraction of absorbed light energy utilized for photochemical charge separation at the PSII reaction centers in the leaf. The application of bacterial isolates led to an almost complete restoration of photosynthetic parameters. Isolate *Bacillus* sp. WS6 proved to be particularly effective: following both single and double treatment, the F_v/F_m and $Y(II)$ values in infected plants approached or even slightly exceeded those of the control. In some variants, $Y(II)$ reached approximately 0.61–0.62, which may indicate not only protection of photosystem II from damage but also stimulation of photochemical processes by *Bacillus* bacteria. These findings are consistent with previous reports demonstrating that plant growth-promoting *Bacillus* strains can mitigate pathogen-induced photodamage [31]. Literature suggests that bacterial colonization often leads to a reduction in reactive oxygen species (ROS) accumulation within the leaf tissues, thereby preserving the structural integrity of the PSII reaction centers [32]. Furthermore, the increase in $Y(II)$ values aligns with studies showing that certain *Bacillus* isolates can upregulate genes associated with the photosynthetic electron transport chain, resulting in higher quantum yield even under biotic stress conditions [33].

Our findings on the low resistance of these strains to various classes of antibiotics are an important indicator of their safety for potential use as biopharmaceuticals. The absence of multidrug resistance reduces the risk of horizontal transfer of resistance genes into soil bacteria.

Dense colonization of the surface, including growth and absorption zones, creates the conditions for mutually beneficial exchange of metabolites and protection from

abiotic and biotic stresses [34,35]. Results obtained using a combination of fluorescence and scanning microscopy techniques clearly demonstrated the close association of bacterial cells with the wheat root system. Colonization indicates the formation of a stable and organized microbial community on the root surface, which is key to realizing both the antagonistic potential of bacteria (biocontrol) and their growth-promoting activity (PGP effects). In this study, we used scanning electron microscopy (SEM) and fluorescent microscopy with the SYTO 9 dye to visualize bacterial cells in a biofilm. However, even such a qualitative (visual) assessment of biofilm formation is important in the context of studying the interaction between plants and microorganisms. It is known that the ability of bacteria to form biofilms on root surfaces is a critical factor for stable colonization and effective biocontrol [36]. The process of biofilm formation in *Bacillus* spp. strains is regulated by a complex hierarchical network of genes, including quorum sensing (QS) systems, which control the coordination of cellular behavior depending on population density [37]. In Gram-positive bacilli, oligopeptide signaling molecules play a key role in activating the expression of genes responsible for the production of extracellular polymeric substances (EPS)—the biofilm matrix [38]. It is likely that the strains we studied utilize similar regulatory cascades, as indirectly indicated by the correlation between their ability to form biofilms *in vitro* and their pronounced antagonistic activity.

The close relationship we have demonstrated has several important implications for wheat physiology. Direct contact between bacteria and the root surface minimizes diffusion losses of metabolites. Bacteria can directly access root exudates, and plants can accumulate mobile nutrients (e.g., iron via siderophores) without intermediaries. The biofilm provides physical protection on the root surface, competing with plant pathogens for attachment sites and nutrients [39]. This explains the previously observed reduction in root rot development in the presence of the studied strains. Close contact (cell-to-cell) is necessary for the transmission of signaling molecules (N-acyl homoserine lactones, AI-2), which regulate gene expression in both bacteria (quorum sensing) and plants (induction of systemic resistance, ISR) [40].

Our results confirm that the phyllosphere isolates studied possess significant biocontrol potential. However, the successful commercialization of such strains requires a comprehensive approach that takes into account not only their antagonistic activity but also production technology, storage stability, and efficacy under real-world field conditions. The development of environmentally safe strategies for controlling plant pathogens requires a transition from laboratory screening to the creation of tailored formulations capable of maintaining bacterial viability throughout the entire growing season. As recently demonstrated in studies on the search for natural biopesticides [41], the most durable protective effect is achieved when using strains with a complex mechanism of action that combines direct antagonism, induction of systemic plant resistance, and competition for ecological niches. In this context, our research is of interest for further development aimed at creating multifunctional consortia or next-generation biopreparations that could become a viable alternative to chemical fungicides in organic and integrated agriculture. In particular, a successful transition to agrochemical models of plant protection requires not only data on antagonistic activity but also an assessment of the economic efficiency of the proposed biotechnological solutions, which should be the subject of subsequent field trials.

Despite the promising results obtained in this study, it should be acknowledged that all experiments were conducted under strictly controlled laboratory conditions (*in vitro*). Although such conditions allow for a clear assessment of the strains' antagonistic potential and their protective effects during the early stages of plant development, they do not fully reflect the complexity of natural agroecosystems. Under real field conditions,

the effectiveness of bacterial bioprotectors can be significantly reduced by a combination of abiotic factors (temperature fluctuations, high humidity, ultraviolet radiation, pH, and soil structure) and biotic factors (competition with the local microbiota, phage pressure, and interaction with soil mesofauna). Furthermore, root system resilience and the production of antimicrobial metabolites in the rhizosphere can vary significantly depending on the physicochemical properties of the soil and the varietal characteristics of the host plant. We view the obtained data as a fundamental basis for subsequent research phases, which should include multifactorial field trials under natural infection conditions, evaluating not only biological efficacy but also the impact of bacterial inoculation on yield and grain quality. Only after successfully passing such a multi-level validation process will it be possible to confidently recommend the studied strains for inclusion in commercial biopesticides.

5. Conclusions

Thus, the strains *Bacillus mojavensis* WP4 and *Bacillus siamensis* WS6, isolated from the phyllosphere of wild wheat (*Aegilops cylindrica*), possess multifunctional growth-promoting properties and exhibit antagonism toward *F. culmorum* in vitro and during the wheat seed germination stage. The combination of these effects (growth promotion and biocontrol) makes them promising candidates for the development of a combined biological product based on them.

Further research should focus, on the one hand, on studying these strains under soil conditions (laboratory experiments) and under field conditions. On the other hand, although our study successfully demonstrated a pronounced antagonistic effect of *Bacillus* spp. strains in vitro and a protective effect on seedlings, the exact chemical nature of the antimicrobial compounds responsible for inhibiting *Fusarium culmorum* remains unknown. The phenotypic obtained data provide a solid foundation for in-depth metabolomic screening. In particular, future studies also will focus on the analysis of secondary metabolites using high-performance liquid chromatography coupled with mass spectrometry to identify specific lipopeptides, siderophores, polyketides, or other low-molecular-weight antifungal compounds produced by the isolates under study. In parallel with metabolomic profiling, whole-genome sequencing (WGS) is planned to map clusters of biosynthetic genes (BGCs) responsible for the production of antibiotics and hydrolytic enzymes. This comprehensive approach, which combines genomic and metabolomic data (pangenomic and metabolomic analysis), will contribute to a deeper understanding of the mechanisms underlying the interactions between microorganisms and pathogens.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16131257/s1>: Figure S1: Experimental Protocol for Stimulating Wheat Growth and Protecting Against a Plant Pathogen; Figure S2: Phylogenetic tree constructed based on 16S rRNA nucleotide sequences for strains WP1, WP3, WP4, WF1, WF2, WF3 and WS6.

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