Article

Enterobacter soli Strain AF-22b-4245: Study of the Genome and the Effect on Wheat Growth

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Abstract: Background: In this work the plant growth-promoting (PGP) qualities of the *Enterobacter soli strain AF-22b-4245* were studied, including screening tests for PGP, whole genome sequencing (WGS) and genome annotation, and greenhouse experiments on wheat. A gene table was formed that allows us to evaluate the potential PGP properties of a microorganism based on the results of genome-wide sequencing. Results: Based on the results of screening tests and genome annotation, it can be concluded that the *E. soli strain AF-22b-4245* strain may have PGP properties, which consist in the ability to survive in arid and saline soils contaminated with copper, arsenic, lead, and chromium soils, form biofilms, produce phytohormones, siderophores, and solubilize phosphorus. Based on the results of experiments on wheat, the *E. soli strain AF-22b-4245* increases the efficiency of mineral fertilizers; this effect persists even in conditions of drought and excess salt. It has been shown that *E. soli A F22b-4245* can compensate for the lack of soluble phosphorus in the mineral fertilizer, probably by solubilizing insoluble forms in the soil.

Keywords: genome annotation; arid soils; saline soils; solubility of phosphates; plant growth-promoting (PGP); whole genome sequencing (WGS); plant growth-promoting bacteria (PGPB)

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1. Introduction

Unfavorable conditions for the cultivation of crops, which include arid and saline soils, reduce the quality and quantity of products or even make these lands unsuitable for agriculture. Among grains, which are vital products for global food security, wheat is one of the most important in the world, and its yield has significantly decreased due to abiotic stress, especially drought and salinization [1]. The introduction of rhizobacteria with plant growth-promoting (PGPR) properties into agricultural systems represents a promising alternative, due to their ability to regulate plant growth and enhance resistance to abiotic stress. These bacteria have the potential to stimulate plant growth and abiotic stress in the soil through the production of bacterial phytohormones, associated metabolites, and significant root morphological changes [2–4].

Among the rhizobacterial species that exhibit plant growth promotion (PGP) properties, *Enterobacter* spp. stand out. These bacteria are known to have a wide range of characteristics related to PGP, including the ability to fix atmospheric nitrogen, dissolve soil phosphates, produce antibiotic substances, release siderophores, phytohormones, and

exopolysaccharides, and increase soil porosity. Additionally, they can produce enzymes such as chitinase, ACC deaminase, and other hydrolytic enzymes [5]. Bacteria belonging to the *Enterobacter cloacae complex*, including *E. mori*, *E. asburiae*, *E. ludwigii*, and *E. sp. J49*, have been shown to promote wheat growth under stressful conditions [6]. The *Enterobacter asburiae A103* strain isolated from the halophyte *Salix linearistipularis* showed high phosphate dissolution activity and effectively stimulated alfalfa growth under alkaline stress [7].

Enterobacter soli was first described as a separate species in 2011 [8]. The *LF7* strain has the ability to destroy kraft lignin. Lignin degradation is a critical and expensive technological step in the production of biofuels; therefore, lignin biodestructors are of particular interest. In 2017, *E. soli* was isolated as an endophytic bacterium of Japanese rice seeds (*Oryza sativa* L.), strain *RWL-4*. Inoculation of rice seeds with this strain contributed to increased root growth, which is associated with the ability to produce indoleacetic acid [9]. In addition, due to the synthesis of exopolysaccharides, *E. soli* is able to sorb chromium effectively [10]. In total, *Enterobacter soli* is a promising microorganism, both for the industrial sector (lignin degradation, 4-VG synthesis) and for agriculture, as a microorganism that improves plant growth and development.

Within the project "All-Russian Atlas of Soil Microorganisms as a Basis for the Search for New Antimicrobial Producers and Enzymes with Unique Properties", we isolated the *Enterobacter soli strain AF-22b-4245*. During the screening process, the strain demonstrated high growth rates in nitrogen-free media and the ability to dissolve phosphates and produce siderophores. The aim of this study was to investigate the properties of the PGPB strain *Enterobacter soli AF-22b-4245*, including screening tests, green house experiments with wheat under standard conditions, and simulations of drought and high salt stress conditions. Additionally, we analyzed the genes associated with plant growth promotion in the genome of *Enterobacter soli AF-22b-4245*.

2. Materials and Methods

2.1. Isolation and Identification

The strain was isolated by the distributed plate method from a soil sample collected in the Bryansk region as part of the civil science project Atlas of Soil Microorganisms of Russia. To accomplish this, 0.1 g of the soil sample was dissolved in 50 mL of sterilized NaCl (0.9%) and thoroughly whipped with a vortex mixer. Then 100 mL was poured into a cup with agar. Isolation was performed on Ashby agar cups with 2% glucose and a small amount of bromothymol blue (BTB) at a temperature of 30 ± 1 °C. After 3-10 days of incubation, colonies with a change in the color of the medium were recorded. To obtain clean colonies, various colonies were selected and purified using the subculture method on appropriate agar media. The morphology and color of the colonies were recorded after 24 h of growth. The identification of bacteria was initially carried out using a Gram staining reaction and then examined using a microscope.

Next, the molecular identification of the isolate was determined on the basis of 16S rRNA sequence analysis. Bacterial isolates were cultured for 48 h, and the DNA of the isolate was extracted according to the procedure described by Sambrook et al. [11]. Amplification of the gene was carried out by PCR using 27F (5-AGAGTTTGATCTTGGCTCAG-3) and 1492R (5-GGT TAC CTT GTT ACG ACT T-3). The purification of PCR products and sequencing were carried out at the SB RAS Genomics Core Facility (http://www.niboch.nsc.ru/doku.php/sequest, accessed on 1 November 2024). The 16S rDNA gene sequences of the bacterial isolates obtained were matched with available gene sequences using BLAST (http://www.ncbi.nlm.nih.gov, accessed on 1 November 2024).

2.2. Quantitative Assessment of Potential Properties That Promote Plant Growth

For each experiment, the microorganism was grown for 24 h at 28 °C in three repetitions in an appropriate medium; a medium without seeding was used as a control. The peptone medium was used to measure the production of gibberellins, ammonium, and siderophores, as well as to determine tolerance to salt, PEG, and heavy metals. A peptone medium with the addition of L-tryptophan (50 mg per 100 mL) was used to measure the production of proline, salicylic acid, and auxin. A Pikovskaya medium was used to measure the solubilization of phosphates. The cells were centrifuged at 10,000 rpm for 10 min, and the culture supernatants was used for measurements. To assess tolerance to salt, PEG, and heavy metals, bacterial suspension was used in the experiment. After adding the appropriate reagents, the wavelength was measured using a spectrophotometer (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA). The average value was calculated from three repetitions.

2.2.1. Solubilization of Insoluble Phosphate

P-solubilization was quantified via the phospho-molybdate blue color method using a spectrophotometer (λ = 882 nm), as described by Murphy and Riley [12]. For quantitative evaluation, a comparison with the standard curve obtained using a standard solution of potassium phosphate was used.

2.2.2. Production of Ammonia

Nesseler's reagent (10 µL) was added to 200 µL of the culture supernatant. The development of a brown to yellow color indicated ammonia production. The absorbance was measured at 450 nm. For quantitative evaluation, a comparison with the standard curve obtained using a standard solution of ammonium sulfate was used.

2.2.3. Production of Indole-3-Acetic Acid

Bacterial isolates were inoculated in sterilized nutrient broth supplemented with 1% tryptophan (precursor for IAA production) then incubated in a shaker for 3 days at 28–30 °C. After the incubation period, the cultures were centrifuged at 10,000 rpm for 10 min before 1 mL of each supernatant was mixed with 2 mL Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) [13]. The mixtures were left at room temperature for 30 min. The development of a pink color indicated the production of IAA, and the quantification of IAA was read at 530 nm. A standard curve was plotted for the quantification of IAA solution and uninoculated medium, with a reagent serving as a control.

2.2.4. Siderophore Production

Culture supernatant and a Chromeazurol S, at a ratio of 1:1, were used as references. The percentage of siderophore units produced was calculated using the following formula: % siderophore unit = $[(Ar - As)/Ar] \times 100$, where Ar = absorbance of the reference at 630 nm and As = absorbance of the sample at 630 nm [14].

2.2.5. Production of Proline

A mixture of acidic ninhydrin reagent (each ml of which contained 0.4 mL of 6 M orthophosphoric acid, 0.6 mL of glacial acetic acid, and 25 mg of ninhydrin), glacial acetic acid, and a sample of culture supernatant in equal proportions was incubated in a water bath at 100 °C for 1 h. The optical density was measured at a wavelength of 520 nm. For quantitative evaluation, a comparison was made with a standard curve obtained by a similar method using standard proline solutions [15].

2.2.6. Production of Salicylic Acid

The synthesis of salicylic acid (SA) in broth culture can be determined by the previously described method [16]. The infusion liquid in the culture was adjusted to pH 2.0 using 1N HCl. After that, the SA was extracted using chloroform (culture supernatant: chloroform; ratio 1:2) by shaking vigorously. For quantitative study, 200 µL of distilled water and 200 µL of 2M $FeCl_3$ were added to 200 µL of the chloroform phase. As a result, a violet Fe–SA complex was formed in the aqueous phase, and the absorption of this complex was measured at a wavelength of 527 nm. SA dissolved in the same growing medium was used as a standard.

2.2.7. Production of Gibberellins

Gibberellic acid was determined based on method described by Abou-Aly et al. [17] as follows: 100 µL of 30% HCl and 100 µL of Folin–Ciocalteu reagent was added to 100 µL of culture supernatant in clear test tube, then 300 µL distilled water was added. The mixture was heated in a thermostat at 100 °C for 5 min then allowed to cool. Intensity of the produced bluish green color was measured at 760 nm using a spectrophotometer. Similarly, color was also developed in standard solution of gibberellic acid (GA_3).

2.2.8. Biofilm Formation

The determination of biofilm formation was carried out on the basis of the previously described method [18]. A total of 200 µL of bacterial suspension in a peptone medium were transferred to the wells of the plate and cultured for 24 h at 37 °C. After that, the contents of the wells were removed, washed twice with a PBS, and, after drying, stained with an aqueous gentian violet (0.1%) for 30 min. Then, the gentian violet was carefully removed and 200 µL of 96% ethanol was added and pipetted. The optical density was estimated at 570 nm.

2.2.9. Salt and PEG Tolerance

Salt tolerance was determined in a peptone medium with the addition of 1%, 5%, 10%, and 15% NaCl. To accomplish this, 50 µL of bacterial suspension was added to 500 µL of medium, incubated for 24 h at 28 °C, then the optical density was estimated at 600 nm. The result was expressed as a percentage of growth relative to the medium without adding salt. PEG tolerance was determined in a similar way. PEG solution was added instead of NaCl.

2.2.10. Tolerance to Heavy Metals

The ability to grow in the presence of heavy metals was determined in a peptone medium with the addition of a salt of the corresponding metal (copper sulfate, lead acetate, zinc chloride, and cadmium chloride). To accomplish this, 50 µL of bacterial suspension was added to 500 µL of medium, incubated for 24 h at a temperature of 28 °C, then the optical density was estimated at 600 nm.

2.3. Genome-Wide Sequencing

2.3.1. WGS Methodology

The genomic library from the isolate DNA sample was prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) reagents with some modifications of the manufacturer's protocol. A total of 1000 ng of genomic DNA was fragmented to 400–500 bp on a Covaris S220 device (Brighton, UK) in 100 µL of sterile water. The fragmented DNA was purified and concentrated using AMPure XP (Beckman Coulter, Brea, CA, USA) magnetic particles; the particles were mixed with DNA in a ratio of 1.6 to 1, respectively. Purified and fragmented DNA was used in the reactions of end completion, adenylation of 3′ ends, and ligation of NEBNext Adapter for Illumina (NEB) adaptors.

DNA with sewn adapters was processed by USER Enzume (NEB) to remove uracil from the adapter and "open" its hairpin structure and purified using AMPure XP (Beckman Coulter) magnetic particles; the particles were mixed with DNA in a ratio of 0.9 to 1, respectively. Then, amplification (3–6 PCR cycles) of the resulting library was performed, during which the adapter sequences were completed, and index sequences were included in them. The qualitative assessment of the obtained libraries was carried out on the Agilent TapeStation 4150 bioanalyzer using the High Sensitivity D5000 ScreenTape and High Sensitivity D5000 Reagents (Agilent, Santa Clara, CA, USA) kits, quantitative—using real-time PCR using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) reagent kit. The resulting genomic library was sequenced on the Illumina NovaSeq 6000 device (San Diego, CA, USA) in the mode of pair-terminal readings 151 + 151 bp.

2.3.2. Assembly De Novo

The quality of the sequencing was assessed using the FastQC v0.12.1 software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ accessed on 2 September 2024). Low-qualities reads were filtered (Q > 28, minlength > 100), and Illumina adapters were trimmed by Trimmomatic v.0.39 [19]. Genome assembly was performed for pair-end reads using the SPAdes v3.15.5 software using parameters -k 21, 33, 55, 77, and -o isolate according to recommendations of developers [20]. The resulting contigues were manually filtered. The filtration threshold for the average coverage level was assessed empirically. First, all the contigues with a coating less than 5 and a length less than 300 were filtered; then the coverage of the remaining ones was manually evaluated. The threshold was considered to be the level of coverage equal to the minimum coverage of extended contigues (70×).

2.3.3. Quality Control and Species Verification

First, the species was determined by blastn tool on 16S_ribosomal_RNA database (ftp://ftp.ncbi.nlm.nih.gov/blast, accessed on 2 September 2024). The best score result was used for the next two rounds of verification of taxonomy. The second step was the analysis using OrthoANI [21]. To compare the de novo genome assembly, sequences of complete genomes with the taxonomy the same to 16S best score analysis were downloaded from the NCBI database. The strains were considered to belong to the same species when the sequences matched by more than 97%. In the third step, the quality of the obtained contigues was evaluated by the Foreign Contamination Screen (FCS) tool. FCS-adapter checks the contigues for service sequences and cuts them out. FCS-GX searches for sequences from a wide range of organisms including bacteria, fungi, protists, viruses, and others to identify sequences that do not look like they are from the best score 16S RNA analysis organism [22]. The type of microorganism was considered certain if it was confirmed by all three methods.

2.3.4. Genome Annotation

The prediction of the open reading frames (ORFs) in the de novo assembly was carried out using GeneMark.hmm software version 3.38 [23]. The resulting list of ORFs was analyzed by software: BlastKoala (KEGG Orthology) [24], InteProScan [25]. Since the fullness of different databases varies, it is advisable to search through all databases in order to identify genes whose protein products are annotated only in some databases. With the help of InterProScan, the assembly was annotated according to the following databases: AntiFam-7.0, CDD-3.20, Coils-2.2.1, FunFam-4.3.0, Gene3D-4.3.0, Hamap-2023_05, MobiDBLite-2.0, NCBIfam-14.0, PANTHER-18.0, Pfam-37.0, PIRSF-3.10, PIRSR-2023_05, PRINTS-42.0, ProSitePatterns-2023_05, ProSiteProfiles-2023_05, SFLD-4, SMART-9.0, SUPERFAMILY-1.75.

2.4. Design of Experiments in Pots

The effect of bacterial suspension on plant growth was studied on "Novosibirsk 31" wheat varieties in a pot experiment in greenhouse conditions. In this experiment, pots with a diameter of 10 cm were used, in which 0.25 kg of soil was placed. Five seeds were placed in each pot at a depth of 2–3 cm. All the selected seeds were surface-sterilized with 1% NaOCl for 90 s and two consecutive rinses in sterile distilled water, followed by air-drying under laminar air flow. Bacterial cultures were grown in 50 mL falcon tubes filled with 10 mL LB broth and were kept in a shaker at 200 rpm for 48 h, then diluted to adjust 10^8 cfu/mL bacterial solutions with sterile, distilled water. The control pots (Water) were irrigated with water. In the case of adding mineral fertilizer (MN), watering was performed with the following solution: N-108, P-39, K-117, Ca-120, Mg-28, S-36,4 (with the addition of chelates of elements Fe, Mn, Zn, Cu, Mo, B). For the stress experimental groups, irrigation was carried out using the following solutions: to simulate drought—12% PEG 6000 (PEG) and to simulate salinization—1.2% NaCl (NaCl). In the case of the simultaneous use of mineral fertilizer and stress (MN-PEG and MN-NaCl), PEG and NaCl solutions were prepared using mineral solution. Irrigation with PEG and NaCl solutions was started two weeks after planting. In all cases, watering was performed as soon as the soil dried out. The experiment was set up as a randomized design, with three biological replications.

2.4.1. Measurements of Plant Parameters

The plant growth lasted for 30 days. During the growing period, the average temperature was 22 °C, and the relative humidity fluctuated was 70–80%. Agronomic parameters such as plant height (cm), root and aboveground biomass (g), dried root, and aboveground biomass (g) were measured after 30 days. The content of chlorophyll a and b, carotenoids were determined as described in [26] (extraction method in 95% ethanol), and the proline content was determined as described in [27] (Colorimetric Assay).

2.4.2. Gene Selection

Based on the analysis of literary sources, we selected 11 wheat genes, the expression of which changes in response to stressful conditions. Wheat transcription factors responding to dehydration and salt stress are as follows: *DREB1* [28], *WRKY26* [29], *TaWRKY71* [30]. Transcription factor ARF2 mediates the expression of genes reacting to the phytohormone auxin [31]. The *CTR1* gene encodes a regulatory component of the ethylene signaling pathway, a phytohormone that modulates the stress response [28]. MARK cascades are involved in signaling hormones, growth factors, and molecular structures associated with damage and convert extracellular stimuli into intracellular reactions, amplifying the transmitting signal [32]. The product of the *ABARE* gene implements the ABA signaling cascade [33]. Enzymes directly involved in the elimination of hydrogen peroxide and superoxides are as follows: POD, CAT 1 [34]. The *TaCKX10* gene encodes cytokine dehydrogenase, which catalyzes the irreversible degradation of cytokines that affect grain yields [35]. The products of lipooxygenase metabolism (encoded by the *LPX* gene) play an important role in the regulation of germination, plant growth and development, aging, damage and stress responses, as well as protection against pathogens [36]. Actin is a constitutive gene as endogenous control.

2.4.3. Measurement of Gene Expression in Wheat

The total RNA of the wheat roots was extracted from different samples using a kit for RNA isolation and purification from plants R-PLANTS (BioLabmix, Novosibirsk, Russia). The concentration and purity of the RNA were verified by measuring the absorbance at 260/280 nm. Reverse transcription and real-time polymerase chain reaction (RT-RT-PCR)

were performed in a one-step method using a kit «BioMaster RT-qPCR SYBR Blue» (Bio-Labmix, Novosibirsk, Russia). The list of primers is presented in Table 1.

Table 1. Structures of primers for evaluation of expression level.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Ref.
DREB2	5′-CGGAGATGCAGCTTCTTGATT-3′	5′-GATCTCGAGCGACGGGTACTT-3′	[23]
CTR1	5′-GCTGCTCTTGTTGAATCCTGTTG-3′	5′-ATCCACAATGCTTGAAAACGAA-3′	[23]
WKY26	5′-TCTTTGGCTTCTCCTTTCACG-3′	5′-TGTTGCTCACTTCTACCACTTG-3′	[26]
TaWKY71	5′-AAACCCGTCATCTCCAAGC-3′	5′-TTGTCCTTGGTCACCTTCTG-3′	[26]
POD	5′-CAGCGACCTGCCAGGCTTTA-3′	5′-GTTGGCCCGGAGAGATGTGG-3′	[29]
CAT1	5′-CCATGAGATCAAGGCCATCT-3′	5′-ATCTTACATGCTCGGCTTGG-3′	[29]
LPX	5′-GAGGTTTTCAAGCGGTTCAG-3′	5′-TTGTGGTCGGAGGTGTTG-3′	[31]
ARF2	5′-TTAAGGTGCGTTGGGATGAG-3′	5′-TTGGCACGAGAAAGAGGAAG-3′	[31]
TaCKX10	5′-GCCATTTCAGTTTCCACGAC-3′	5′-TCAAGAACACATGCCTCACG-3′	[31]
MAPK	5′-CCTACTGGGTCGTTTACTTGC-3′	5′-CGAAATTGGATGCCTTGATGG-3′	[31]
ABARE	5′-TTACACCGTGGAGCTTGAAG-3′	5′-TTCACGTTCTCCTTGGACTG-3′	[33]
Actin	5′-TGCCCATTTACGAAGGATACG-3′	5′-GTGTTGGGTTCACAATGTCG-3′	[31]

A thermal cycler CFX C1000 (BioRad, Hercules, CA, USA) was implemented using a cycling program of 95 °C for 180 s, 45 cycles of 95 °C for 5 s, 60 °C for 5 s, and 20 s at 72 °C. The expression of genes under study was computed by using threshold (Ct) value for each gene normalized against the Ct for actin from wheat which was used as the constitutive reference transcript. The relative expression levels of all samples were calculated and analyzed based on the $2^{-\Delta\Delta CT}$ method [37].

2.4.4. Statistical Analysis

Comparing the groups for statistical differences in the data, the significance was tested using ordinary one-way ANOVA analysis and Tukey's multiple comparisons test by GraphPad Prism 10 (https://www.graphpad.com/features, accessed on 12 August 2024). A principal component analysis (PCA) was performed to analyze the relationships between soil isolates and parameters measured by tests. PCA was performed using the R procomp function with standard parameters (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/prcomp, accessed on 12 May 2023). The results are presented on a two-dimensional graph, the axes of which are the first two main components: PC1 and PC2. The interpretation of the graph is based on the clustering of samples into groups. To visualize the numerical change in the level of gene expression, a clustered heat map was built using pheatmap v. 1.0.12 function in R package [25].

3. Results

The *AF-22b-4245 strain* is a gram-negative rod. It forms rounded, small, shiny cream-colored colonies when growing on an agarized nutrient medium, of the following composition (g/L): glucose—10, agar—15, $FeSO_4 \cdot 7H_2O$—0.05, KH_2PO_4—0.5, $CaCl_2$—0.1, $MgSO_4 \cdot 7H_2O$—0.1, NaCl—0.1, $CaCO_3$—0.5, a solution of trace elements—2.0. Well-isolated colonies up to 3 mm in size are formed after 3 days of cultivation at a temperature of 28–37 °C.

3.1. Screening on Titer Plates

After isolation, the strain was initially routinely screened in titer plates for PGRB properties and stress resistance, which included drought tests, salinity tests, survival in

the presence of heavy metals, production of phytohormones and ammonium, the ability to form biofilms, and solubilize phosphates (Table 2).

Table 2. The results of the screening on titer plates.

Stress Tolerance				
	1%	**5%**	**10%**	**15%**
PEG NaCl	100.00 ± 21.93 99.99 ± 27.51	88.70 ± 2.55 44.99 ± 1.99	58.82 ± 0.95 0.40 ± 0.05	51.94 ± 3.88 0.0 ± 0.03
Metals	Cu 51.49 ± 5.48	Pb 62.33 ± 0.19	Zn 19.25 ± 1.51	Cd 0.74 ± 0.10
Production of phytohormones, proline, siderophores, ammonium, biofilms and soluble forms of phosphates				
IAA, μg/mL	GA, μg/mL	SA, μg/mL	Prolin, μg/mL	Ammonia, μg/mL
79.74 ± 17.54	2.42 ± 0.20	0.55 ± 0.40	45.61 ± 20.10	24.26 ± 13.01
Biofilm formation	Solubilization of phosphates, μg/ml		Siderophores, %	
0.12 ± 0.01	134.69 ± 14.8		31.31 ± 5.74	

It was found that the *E. soli strain AF-22b-4245* tolerated drought conditions well, was able to survive at a substrate-metric potential of −0.30 MPa (PEG concentration of 15%), and tolerated salinity well (up to 5% of NaCl). Tests on the ability to survive in an environment with heavy metals showed that the *E. soli strain AF-22b-4245* was able to grow in the presence of copper and lead. The *E. soli strain AF-22b-4245* is a good producer of phytohormones (IAA, gibberellins), ammonium, and siderophores. In addition, high rates of phosphate solubilization were recorded.

3.2. Whole-Genome Sequencing

Whole-genome sequencing was performed, followed by de novo genome assembly and annotation in order to identify genes that are responsible for the presence of traits useful for agriculture.

3.2.1. Identification of the Species

The species was determined based on several indicators: the results of BLAST 16S rRNA, the results of OrtoANI analysis (Figure 1), and the results of FCS-GX analysis. The best result of 16S—*Enterobacter soli ATCC BAA-2102 strain LF7* 16S ribosomal RNA, partial sequence, the maximum value of OrtoANI alignment for whole genomes was obtained for the *Enterobacter soli strain LF7a* (97.44%). FCS-GX verification showed that all extended contigues belonged to *Enterobacter soli*. Consequently, the bacterium was identified as *E. soli*. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBHGCI000000000. The version described in this paper is version JBHGCI000000000.1.

3.2.2. Genome Annotation

The general characteristics of the genome are summarized in Table 3. Due to the different content of databases with information about genes, we carried out annotation in two stages. First, based on the literature data, we formed a list of genes that are responsible for the presence of traits useful for agriculture. The genes were divided into groups: (1) biofilm formation, (2) nitrogen metabolism, (3) siderophore production, (4) phytohormone production, (5) phosphate solubilization, (6) heavy metal bioremediation, and (7) oxidative stress response genes. The second stage was to evaluate the presence of

these genes in *Enterobacter soli strain AF-22b-4245*, correlate them with screening test data, and predict the presence of PGBP properties.

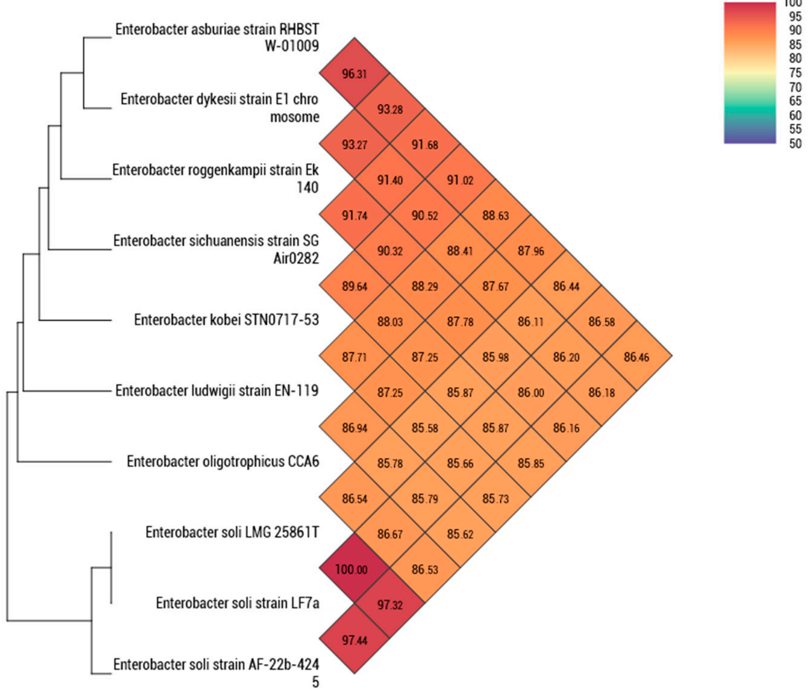


Figure 1. Alignment to the complete genomes of *Enterobacter* sp.

Table 3. General characteristics of genome of *Enterobacter soli strain AF-22b-4245* by PGAP.

NCBI GenBank	*Enterobacter soli Strain AF-22b-4245*
Species	*Enterobacter soli*
BioProject	PRJNA1146973
BioSample	SAMN43174172
Accession	JBHGCI000000000
Total base	4,973,019
GC (%)	53.6%
Coverage, min	70×
Genes (total)	4785
CDSs (total)	4698
Genes (coding)	4645
CDSs with protein	4645
Genes (RNA)	87
rRNAs	1, 1, 2 (5S, 16S, 23S)
complete rRNAs	1, 1, 1 (5S, 16S, 23S)
partial rRNAs	1 (23S)
tRNAs	75
ncRNAs	8
Pseudo genes	53
CDS (without protein)	53
Pseudo Genes (ambiguous residues)	0 of 53
Pseudo Genes (frameshifted)	14 of 53
Pseudo Genes (incomplete)	35 of 53
Pseudo Genes (internal stop)	11 of 53
Pseudo Genes (multiple problems)	6 of 53
Pseudo Genes (ambiguous residues)	0 of 53

Biofilm formation. *E. soli strain AF-22b-4245* possesses the entire spectrum of biofilm formation genes (Table S1. (Sheet "Biofilm formation")), annotated in KEGG, with the exception of *pgaABCD* and some small regulatory RNAs.

Nitrogen metabolism. Nitrogen metabolism is usually divided into several key processes: nitrogen fixation, dissimilatory nitrate reduction, assimilatory nitrate reduction, nitrification, denitrification, and anammox (Table S1. (Sheet "Nitrogen metabolism")). The strain does not possess a nitrogenase cascade of genes of any of the systems described in the literature (nif (Mo-Fe), vnf (V-Fe), and anf (Fe-Fe)) [38]. Despite the fact that *AF-22b-4245* has the *nifS*, *nifU*, and *nifJ* genes, this fact does not indicate the possibility of nitrogen fixation, since the key and critically necessary genes for this are the catalytic domain genes, usually located in a row in the genome (*nifHDK*) and the genes of the biosynthetic domain (*nifENB*).

The *E. soli strain AF-22b-4245* has the genes of dissimilatory nitrate reduction (*narGHI*, *NirBD*), assimilatory nitrate reduction (*nasABCDEF*), and therefore has the ability to convert nitrate into ammonia. *E. soli strain AF-22b-4245* also possesses the glutamate dehydrogenase gene (*gudD* [EC:1.4.1.2]), carrying out the reaction H_2O + L-glutamate + NAD^+ = 2-oxo-glutarate + H^+ + NADH + $NH_4{}^+$ [39], one of the products of which is ammonia. This is in good agreement with screening tests that show high ammonia production.

Siderophore production. Iron is involved in several key cellular processes of microorganisms, including amino acid synthesis, oxygen transport, respiration, nitrogen fixation, methanogenesis, the citric acid cycle, photosynthesis, and DNA biosynthesis. The concentrations of iron in the extracellular medium are low (10–18 microns) and limited by the insolubility of $Fe(OH)_3$ [40]. To overcome the deficiency, some microorganisms synthesize siderophores, small molecules that specifically bind trivalent iron with high affinity. Siderophores have been classified based on their structural features and functional groups, forming iron chelates that are divided into three main classes: catecholates (also called phenocatecholates or siderophores derived from salicylate), hydroxamates, and carboxylates. In addition, there are siderophores of a mixed type (containing more than one of the above-mentioned groups) [41]. In bacteria, siderophores are synthesized by the products of biosynthetic cluster (BGC) genes. As a rule, BGCs are located in the genome in the form of continuous clusters, and their genes encode all the enzymes necessary for the synthesis of a secondary metabolite. All genes are organized as an operon and expressed together. Enterobactin synthesis is carried out by *entABCDEF* genes [42], pyochelin synthesis by *pchDHIEFKCBA* genes, and mycobactin synthesis by *MbtA-J* genes [41].

Additionally, there are receptors on the surface of the membrane of microorganisms that are responsible for the recognition and transfer of iron-bound siderophores from the extracellular space inside: *fepABCDG* complexes (capture of ferroenterobactin) [43], *yclNOPQ* (capture of petrobactin) [44], *fecABCDE* (Fe(3+) dicitrate transport) [45], and *fhuABCDE* (ferric hydroxamate transport) [46]. The outer membrane of gram-negative cells does not have a proton driving force that would provide the energy necessary for active transport; therefore, tonB, ExBB, and exbD proteins perform this role [47]. Cases have been described when organisms used xenosiderophores or siderophores produced by other microorganisms to compensate for their needs [48]. By summarizing the above, genes for siderophore synthesis (*entABCDEFDS*) and membrane transport (*fepABCDG*, *fes*, *yclNOPQ*, *fecABCDER*, *fhuABCDEF*, *fpvA*, *exbBD*, *tonB*) were selected to annotate genomes with respect to the ability to absorb iron (Table S1 (Sheet "Siderophore")). The *E. soli strain AF-22b-4245* has enterobactin synthesis genes and all transport genes except *yclNOPQ*, which is consistent with the screening results.

Production of phytohormones.

Production of salicylic acid. Salicylic acid in a bacterial cell is obtained from chorismate in two stages. The first stage is to convert chorismate into isochorismate by the enzyme

isochorismate synthetase (*pchA*, *entC*, *menF*), and the second—isochorismate pyruvate lyase converts isochorismate into pyruvate and salicylate. In *Mycobacterium* and *Yersinia*, chorismate is converted to salicylate by a single bifunctional enzyme encoded by *mbtI* and *Irp9*, respectively. As a rule, salicylate is further assimilated by bacteria into the siderophore framework of catecholate siderophores (pyochelin, bacillibactin, and mycobactin). The *E. coli strain AF-22b-4245*, according to the results of the screening test, produces salicylic acid in a very small amount (0.55 ± 0.40 μg/mL), which is consistent with the fact that its genome lacks a biosynthetic cluster of genes containing both isochorismate synthetase and isochorismatliase. The *entABCDEFDS* gene cluster contains only the isochorismate synthetase *entC*. Most likely, the screening method detects not only salicylic acid, but also its metastable precursor isochorismate [49]. Based on the above, it can be assumed that this method of determining salicylic acid may indirectly indicate the potential presence of siderophore synthesis genes, the precursor of which is salicylic acid and isochorismate.

Production of indoleacetic acid. The ability to synthesize indoleacetic acid is a well-studied feature of many PGPB bacteria. Indole-3-acetic acid (IAA) is the most important phytohormone in the process of plant growth and development [50]. It has been experimentally shown that IAA synthesized by bacteria of the chickpea rhizosphere enhances root growth [51].

As a rule, IAA is synthesized in two ways: Trp-dependent and Trp-independent. However, the Trp-independent way has not yet been identified in bacteria. The four main Trp-dependent ways of IAA synthesis in bacteria are described in the publication by Zhang et al. [50]. We highlighted the KEGG tryptophan metabolic way with arrows of different colors for the ease of perception of information about the presence of the necessary genes in the bacterium (Figure 2). The first way (crimson) is through indole-3-pyruvate, the second (green) is through tryptamine, the third (orange) is through indole-3-acetonitrile, and the fourth is through indole-3-acetamide. A bacterium may have several synthesis ways, but the issue of regulation and functional redundancy is still open [52].

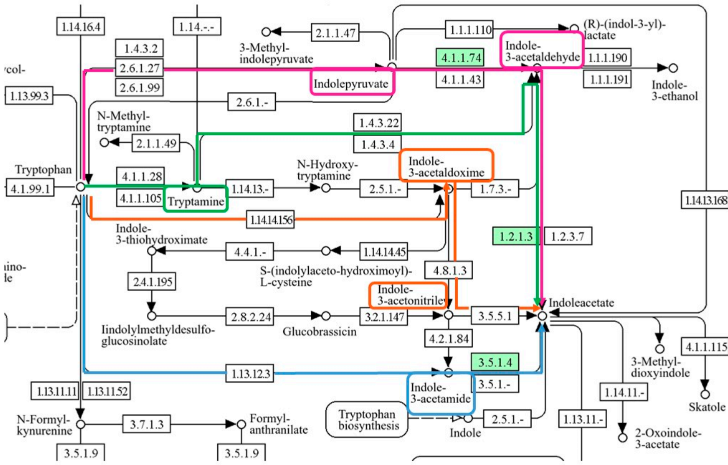


Figure 2. Pathways of indoleacetic acid synthesis using the example of the KEGG Tryptophan metabolism map (map00380) for *E. soli* AF-22b-4245. The first pathway (crimson) is through indole-3-pyruvate, the second (green) is through tryptamine, the third (orange) is through indole-3-acetonitrile and the fourth is through indole-3-acetamide (blue). Adapted from P. Zhang et al. [44].

Enterobacter soli strain AF-22b-4245 possesses genes for the synthesis of IAA through indole pyruvate *ipdC*, *ALDH* (Figure 2, Table S1. (Sheet "Phytohormones")). Despite the fact that the annotation of KEGG genes of the first step in indole pyruvate (transfer of the amino group from tryptophan to α-ketoglutarate using aminotransferase to form indole-3-pyruvate and L-glutamate) was not revealed, screening tests showed that *E. soli strain*

AF-22b-4245 had the ability to synthesize IAA. Experiments have been described in which the metabolism of aromatic amino acids in many lactic acid bacteria is initiated by aminotransferases with broad substrate specificity [52], probably in *E. soli strain AF-22b-4245*; the first step of indole pyruvate synthesis of IAA is also carried out by some aminotransferase of broad specificity, not shown on the Tryptophan metabolism map (map00380).

Production of gibberellins. Genome annotation did not reveal gibberellin synthesis genes in the studied strains (Table S1 (Sheet "Phytohormones")), although a screening test showed the synthesis of gibberellins for *E. soli strain AF-22b-4245*. Perhaps the test detected molecules with a steric structure similar to gibberellins.

Production of ethylene. Rhizospheric bacteria can stimulate plant growth by reducing the level of the plant hormone ethylene through the deamination of its immediate precursor 1-aminoacyclopropane-1-carboxylate. The reaction is carried out by 1-aminocyclopropane-1-carboxylate deaminase encoded by the *accD* gene. The studied strain of *E. soli strain AF-22b-4245* possess the *accD* gene (Table S1 (Sheet "Phytohormones")).

Solubility of phosphates. Phosphorus is contained in two forms in the soil: mineral and organic (mainly in the form of inositol phosphate (soil phytate)). The cell can take several forms, but most of it is absorbed in the form of phosphate. Thus, microorganisms can increase the amount of phosphorus available to plants in two ways.

The first way is the dissolution of mineral phosphorus due to the secretion into the external environment of low molecular weight organic acids such as citric, oxalic, gluconic, 2-ketogluconic, lactic, malic, amber, tartaric, and acetic. Citric acid is one of the most effective organic acids for the solubilization of minerals P from various phosphate rocks due to its good chelating and strongly acidic properties [53]. It has been experimentally shown that 5–10 mm of citric acid is sufficient to release P, while most other acids are required at concentrations of 50–100 mmol/L [53].

The second way is the release of phosphorus from organic compounds by three groups of enzymes: (1) non-specific phosphatases that dephosphorylate phosphoester or phosphoanhydride bonds in organic matter, (2) phytases that specifically cause the release of phosphorus from phytic acid, and (3) phosphonatases and C–P-lyases, enzymes that cleave C–P of organophosphorus compounds. The main proportion is released due to the work of acid phosphatases and phytases due to the predominant presence of their substrates in the soil [54].

The genes whose products carry out the described processes are summarized in the table (Table S1 (Sheet "Phosphate solubilizing")). The *E. soli strain AF-22b-4245* possesses citric acid (*citC*) and fumaric acid (*dcuS*) secretion genes. An additional mechanism of phosphorus solubilization may be the chelation of iron with siderophores from iron phosphates [55], and *E. soli strain AF-22b-4245* has genes for the synthesis of enterobactin. This fact is in good agreement with the results of the screening test, in which the strain showed very high rates of phosphate solubilization.

Bioremediation of heavy metals. Bioremediation includes processes as a result of which the natural environment, modified by pollutants, returns to its original state. Interactions between metals and microbes are usually classified into (1) the conversion of heavy metals into less toxic compounds, (2) cytosol-binding compounds can bind and neutralize metals inside the cell, and (3) sorption by the cell membrane [56].

The strain has *arsC* (arsenic reduction) and *chrR* (chromium reduction) reductase genes. The strain also has a copper efflux system, which is consistent with the results of a screening test for survival in the presence of copper (Table S1 (Sheet "Heavy metall")).

Genes for the response to oxidative stress. Superoxide dismutase (*sodA*, *sodB*) and catalase are key bacterial proteins in the fight against oxidative stress. They are endogenous enzymatic antioxidants: superoxide dismutase catalyzes the reaction of superoxide

utilization to form hydrogen peroxide, and catalase detoxifies hydrogen peroxide. These enzymes are regulated by transcription factors (redox sensors) of oxidative stress OxyR and soxRS [57]. BLAST analysis has shown that 176 α-, β-, δ-, and γ-proteobacteria and actinobacteria have *soxR* homologues, while *soxS* is found only in the Enterobacteriaceae family [58]. The *E. soli strain AF-22b-4245* possesses *soxR* and *soxS*, *CatB*, *sodA*, and *sodB* (Table S1 (Sheet "Oxidoreductases")).

The *E. soli strain AF-22b-4245* also has the *OxyR* gene. *OxyR* is a transcription factor, a hydrogen peroxide sensor. It activates the expression of H_2O -induced genes [59].

By summarizing the results of screening tests with the results of genome annotation, it can be concluded that the *E. soli strain AF-22b-4245* may have PGBP properties, which consist in the ability to survive in arid and saline soils contaminated with copper, arsenic, lead, and chromium soils, form biofilms, produce phytohormones, siderophores, and solubilize phosphorus.

3.3. Experiments on Plants

In order to test in practice, the degree of manifestation of the PGBP properties of the *E. soli strain AF-22b-4245*, several experiments were conducted on wheat since wheat is one of the main grains in Russia. The experiments were designed to evaluate the properties under different stress conditions and without it, as well as the ability to increase the efficiency of mineral fertilizers. The following experiments were carried out: (1) absence of stress factors, (2) imitation of arid soil or saline soil, and (3) lack of nutrients.

3.3.1. Absence of Stress Factors

The purpose of the first experiment was to evaluate the growth qualities of wheat when watering with bacterial suspension in two different ways: application on a leaf and watering under the root. Bacterial suspension treatment was carried out one time per week, a total of four times per experiment. Additionally, in this experiment, the ability of the *E. soli strain AF-22b-4245* to increase the efficiency of mineral fertilizers was evaluated. Watering of plants with water or a solution of mineral fertilizers was carried out daily. The results of the experiment are summarized in Table 4 and Figure 3.

Table 4. The results of the experiment in the absence of stress factors.

Treatment	Bacteria Addition	Plant Height (cm)	Leaf Fresh Weight (g plant^{-1})	Root Fresh Weight (g plant^{-1})	Leaf Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})
CK	no	25.63 ± 1.83	0.61 ± 0.13	0.61 ± 0.08	0.20 ± 0.04	0.29 ± 0.03
CK	root	26.13 ± 1.05	0.75 ± 0.04	0.84 ± 0.06 *	0.28 ± 0.05	0.26 ± 0.05
CK	leaf	25.69 ± 0.94	0.69 ±0.06	0.65 ± 0.13	0.28 ± 0.03	0.24 ± 0.02
MF	no	27.69 ± 1.13	0.75 ± 0.07	0.72 ± 0.05	0.28 ± 0.04	0.32 ± 0.06
MF	root	31.60 ± 1.14 ****	1.04 ± 0.09 ****	0.88 ± 0.08 **	0.36 ± 0.04 ***	0.41 ± 0.07 *
MF	leaf	30.06 ± 1.03 ***	0.96 ± 0.14 ***	0.88 ± 0.14 **	0.36 ± 0.04 ***	0.38 ± 0.06

Data represent the mean ± SE (n = 4). CK, control not treated with MF; MF, treated with mineral fertilization. Statistically significant values are marked by asterisk: *—level $p < 0.05$, **—level $p < 0.01$, ***—level $p < 0.001$, ****—level $p < 0.0001$. The CK group without bacteria addition was used as a control comparison group.

According to the obtained data, it can be concluded that the *E. soli strain AF-22b-4245* has a positive effect on the growth and development of wheat. The most versatile increase in growth characteristics was obtained for a combination of watering with bacterial suspension under the root in the presence of mineral fertilization. Thus, it was shown that the *E. soli strain AF-22b-4245* increased the efficiency of mineral fertilizers. Since, when applied under the root, a statistically significant increase was recorded in all indicators, except for the weight of dried roots, as opposed to applying on a leaf; watering under the

root was considered the most optimal form of application. In further experiments in this work, application under the root was used.

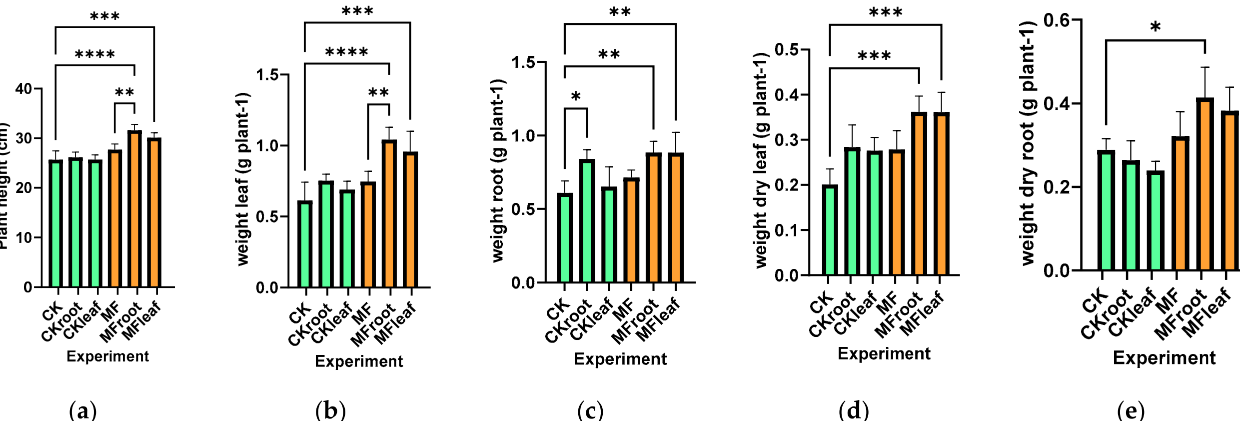


Figure 3. Diagrams of economic indicators of plants after treatment with bacteria and mineral fertilizer: (**a**) height of the plant, (**b**) the weight of the aboveground part of the plant, (**c**) the weight of the underground part of the plant, (**d**) the dry weight of the aboveground part of the plant, (**e**) the dry weight of the underground part of the plant. Statistically significant values are marked by asterisk: *—level $p < 0.05$, **—level $p < 0.01$, ***—level $p < 0.001$, ****—level $p < 0.0001$.

3.3.2. Growing Wheat Under Stress

Due to the fact that in screening tests, the *E. soli strain AF-22b-4245* demonstrated good survival in the presence of PEG and NaCl, the aim of the next experiment was to assess whether the presence of *E. soli strain AF-22b-4245* has a positive effect on the growth and development of wheat in conditions of drought and excess salt. Drought simulation in experimental conditions is carried out by exposing plants to substances that change the osmotic potential in plant cells, for example, with a solution of polyethylene glycol [60]. Imitation of saline soil under experimental conditions was carried out by exposing plants to NaCl through watering with a 1% salt solution (150 mM sodium chloride solution). A brief scheme of the experiment with a stress source was the following: for 14 days, the plants were grown under standard conditions, and from the third week, the plants were stressed by watering with a 20% PEG-6000 solution in water or a 1% salt solution (150 mM sodium chloride solution). On day 30, wheat was dug up, and growth characteristics were evaluated (plant height, weight of the aboveground part and root before drying, and weight of the aboveground part and root after drying). To assess the stress level to which wheat was subjected, the ratio of chlorophyll A to chlorophyll B, the level of proline (Table 5, Figures 4 and 5), and the expression level of genes involved in the stress response in plants were measured.

According to the obtained results, the application of bacterial suspension or mineral top dressing statistically significantly improved almost all morphometric parameters, but the best effect was achieved with complex application—the application of mineral fertilizers combined with irrigation with bacterial suspension, therefore, the comparison values are presented for the conditions of mineral fertilizers and the bacterial suspension against watering with water (CK group).

In the absence of stressful conditions, the following indicators reached the level of statistical significance: plant height (36.7 ± 1.57 cm vs. 22.1 ± 3.13, $p < 0.0001$); the weight of the aboveground part of the plant before drying (1.11 ± 0.18 vs. 0.49 ± 0.11, $p < 0.0001$) and after drying (0.49 ± 0.18 vs. 0.14 ± 0.01, $p < 0.001$); and the weight of roots before drying (0.97 ± 0.16 vs. 0.45 ± 0.10, $p < 0.001$) and after drying (0.33 ± 0.13 vs. 0.13 ± 0.03, $p < 0.001$).

Table 5. Growth and biochemical characteristics of wheat when grown under stress conditions (drought, salinity).

Stress	Treatment	Bacteria Addition	Plant Height (cm)	Leaf Fresh Weight (g plant^{-1})	Root Fresh Weight (g plant^{-1})	Leaf Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	ChlA/ChlB	Proline
No	CK	No	22.1 ± 3.13	0.49 ± 0.11	0.45 ± 0.10	0.14 ± 0.01	0.13 ± 0.03	1.75 ± 0.08	0.23 ± 0.07
No	CK	Yes	25.1 ± 1.03	0.78 ± 0.09	0.70 ± 0.04	0.21 ± 0.01	0.21 ± 0.06	1.66 ± 0.03	0.33 ± 0.04
No	MF	No	28.1 ± 1.63 *	0.80 ± 0.12	0.69 ± 0.13	0.28 ± 0.10	0.20 ± 0.03	1.85 ± 0.07	0.33 ± 0.04
No	MF	Yes	36.7 ± 1.57 ****	1.11 ± 0.18 ****	0.97 ± 0.16 ***	0.49 ± 0.18 ****	0.33 ± 0.13 **	1.26 ± 0.36	0.45 ± 0.08
NaCl	CK	No	21.8 ± 1.91	0.38 ± 0.06	0.47 ± 0.08	0.14 ± 0.04	0.13 ± 0.02	1.96 ± 0.08	0.20 ± 0.06
NaCl	CK	Yes	25.5 ± 1.81	0.69 ± 0.11	0.73 ± 0.09	0.18 ± 0.01	0.21 ± 0.01	1.50 ± 0.42	0.47 ± 0.18 *
NaCl	MF	No	26.2 ± 1.14	0.66 ± 0.13	0.53 ± 0.13	0.23 ± 0.03	0.17 ± 0.02	1.61 ± 0.05	0.36 ± 0.03
NaCl	MF	Yes	28.5 ± 2.61 *	0.79 ± 0.18 **	0.76 ± 0.10	0.36 ± 0.03 *	0.17 ± 0.02	1.66 ± 0.24	0.37 ± 0.05
PEG	CK	No	21.3 ± 0.46	0.31 ± 0.05	0.32 ± 0.08	0.11 ± 0.01	0.16 ± 0.03	1.93 ± 0.93	0.23 ± 0.06
PEG	CK	Yes	24.8 ± 2.33	0.59 ± 0.08 *	0.53 ± 0.09 *	0.17 ± 0.03	0.29 ± 0.03	1.39 ± 0.40	0.42 ± 0.18
PEG	MF	No	28.7 ± 2.70 **	0.64 ± 0.08	0.43 ± 0.15	0.22 ± 0.02	0.16 ± 0.02 *	0.98 ± 0.18 **	0.58 ± 0.08 ***
PEG	MF	Yes	29.0 ± 0.90 **	0.74 ± 0.08 **	0.63 ± 0.13	0.25 ± 0.02	0.26 ± 0.03	1.21 ± 0.22 *	0.48 ± 0.07 *

Data represent the mean ± SE (n = 3). CK, control not treated with MF; MF, treated with mineral fertilization; NaCl—stress caused by adding NaCl (salinity); PEG—stress caused by adding PEG (drought). Statistically significant values are marked by asterisk: *—level $p < 0.05$, **—level $p < 0.01$, ***—level $p < 0.001$, ****—level $p < 0.0001$. Comparison with the control group (CK + No bacteria addition) was carried out separately for different stress condition.

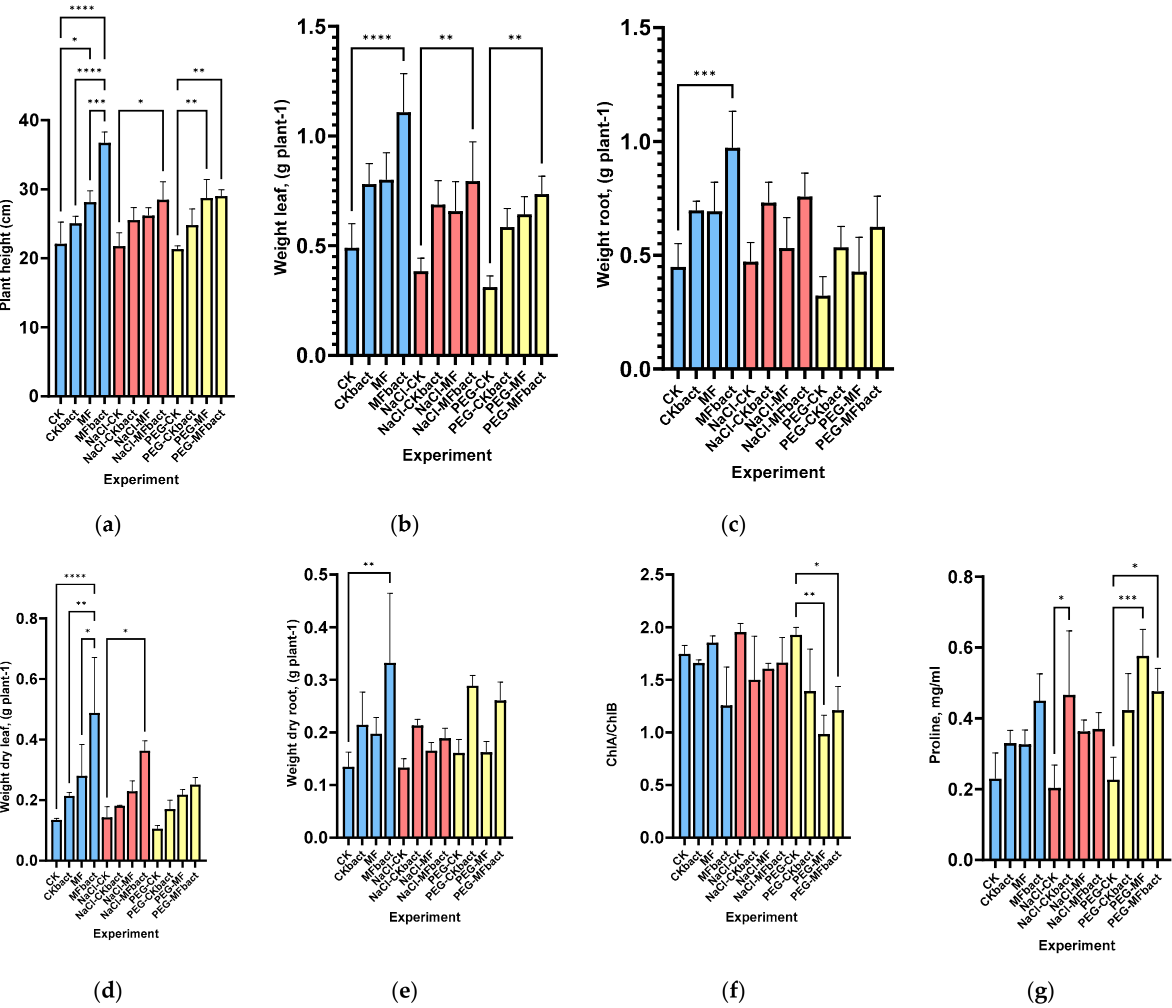


Figure 4. Diagrams of economic indicators of plants after treatment with bacteria and mineral fertilizer: (**a**) height of the plant, (**b**) the weight of the aboveground part of the plant, (**c**) the weight of the underground part of the plant, (**d**) the dry weight of the aboveground part of the plant, (**e**) the dry weight of the underground part of the plant, (**f**) ratio of chlorophyll A to B, (**g**) level of proline. Statistically significant values are marked by asterisk: *—level $p < 0.05$, **—level $p < 0.01$, ***—level $p < 0.001$, ****—level $p < 0.0001$.

Under conditions of salt stress, the effect of the combined use of mineral fertilizers and bacterial suspension was maximal, and statistically significant differences were achieved in the following indicators: plant height (28.5 ± 2.61 cm vs. 21.3 ± 0.46 cm, $p = 0.02$) and the weight of the aboveground part of the plant before drying (0.79 ± 0.18 vs. 0.38 ± 0.06, $p < 0.01$) and after drying (0.36 ± 0.03 vs. 0.14 ± 0.04, $p = 0.002$).

In drought conditions, the effect of the combined use of mineral fertilizers and bacterial suspension was also maximal: plant height (29.0 ± 0.90 cm vs. 20.5 ± 2.13 cm, $p < 0.01$); the weight of the aboveground part of the plant before drying (0.74 ± 0.08 vs. 0.31 ± 0.05, $p < 0.01$); the ratio of chlorophyll A to B (1.21 ± 0.22 vs. 1.93 ± 0.93, $p = 0.02$); and the level of proline (0.48 ± 0.07 vs. 0.23 ± 0.06, $p < 0.05$).

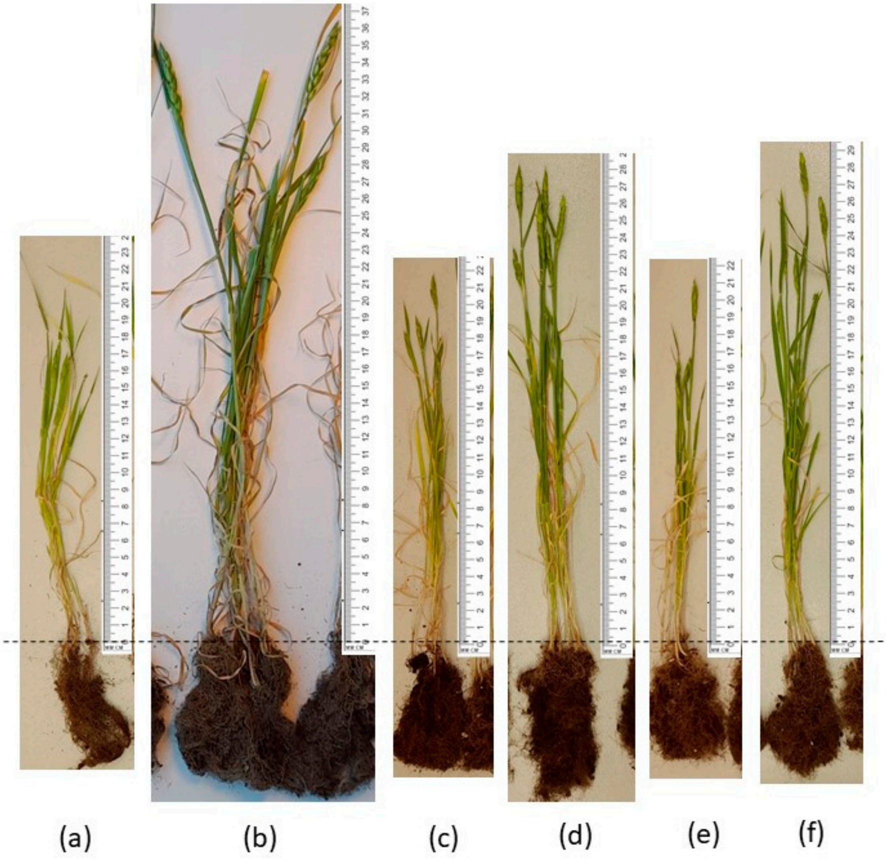


Figure 5. Photos of wheat after 30 days of growing under stress (drought and salinity) and without it. (**a**) no stress, CK, no bacteria addition, (**b**) no stress, MF, add bacteria, (**c**) NaCl, CK, no bacteria addition, (**d**) NaCl, CK, add bacteria, (**e**) PEG, CK, no bacteria addition, (**f**) PEG, CK, add bacteria.

In order to visualize the consistency of changes in growth and biochemical characteristics depending on wheat growing conditions, a PCA analysis was performed (Figure 6).

According to the results obtained, the first component 1 (PC-1) describes 68.4% of the variability of the initial indicators, and the second—22.1%. In total, the PC-1 and PC-2 sample clustering graph takes into account 90.5% of the variability of the initial parameters. The graph clearly visualizes three clusters according to growing conditions: (1) without the addition of mineral fertilizers and/or *E. soli strain AF-22b-4245*, (2) a group of samples in the center, and (3) a Water-MF-1156 sample was laid separately. This corresponds to the fact that the highest rates of growth and weight were in plants without stress with combined fertilization, and the lowest rates were in plants without any fertilization. It can be seen that PC-1 coefficients for all initial characteristics are approximately equal in modulus, which means their contribution is comparable. The negative value of the ratio of chlorophyll A to B is consistent with its physical meaning—the more chlorophyll B is

destroyed (reflects stress), the lower the coefficient. In total, plants respond with increased growth in response to fertilization, even under stress. Within the second cluster, a subgroup of green samples that were exposed to drought conditions can be distinguished. According to PC-2 coefficients (maximum negative values in modulus), clustering in this group is provided by indicators of the ratio of chlorophyll A to B and the level of proline. This is consistent with the biological meaning, since compensation for the increased external osmotic pressure of the plant is carried out due to the accumulation of proline [61].

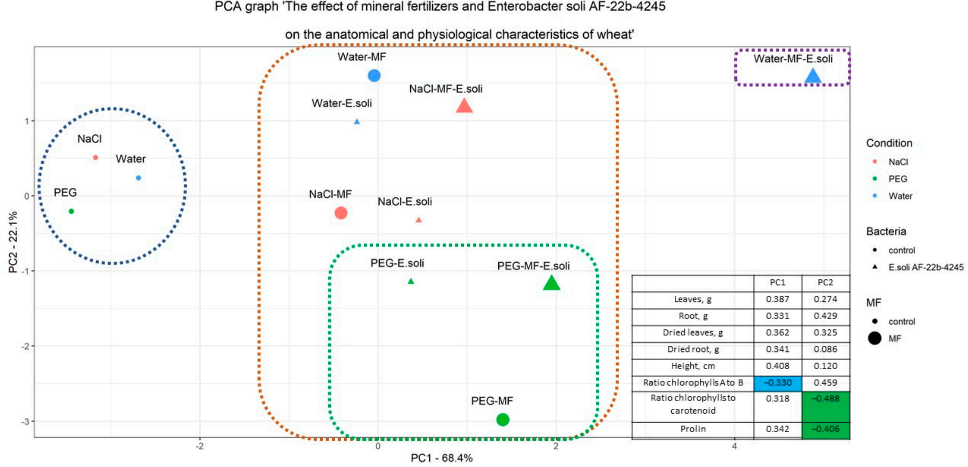


Figure 6. PCA on growth and biochemical characteristics under different wheat growing conditions.

Additionally, the expression levels of wheat genes, which according to the literature data are involved in the stress response: *ABARE*, *ARF2*, *CAT*, *CKX10*, *CTR1*, *DREB*, *MAPK*, *LPX*, *POD*, *WKY26*, and *WKY71* (Figure 3), were evaluated. The level of $2^{-\Delta\Delta CT}$ was determined relative to the level of the constitutively expressed actin gene. It was found that the addition of *E. soli strain AF-22b-4245* suspension led to a decrease in the expression level of all wheat stress genes in conditions of drought and increased soil salinity (Figure 7a,b).

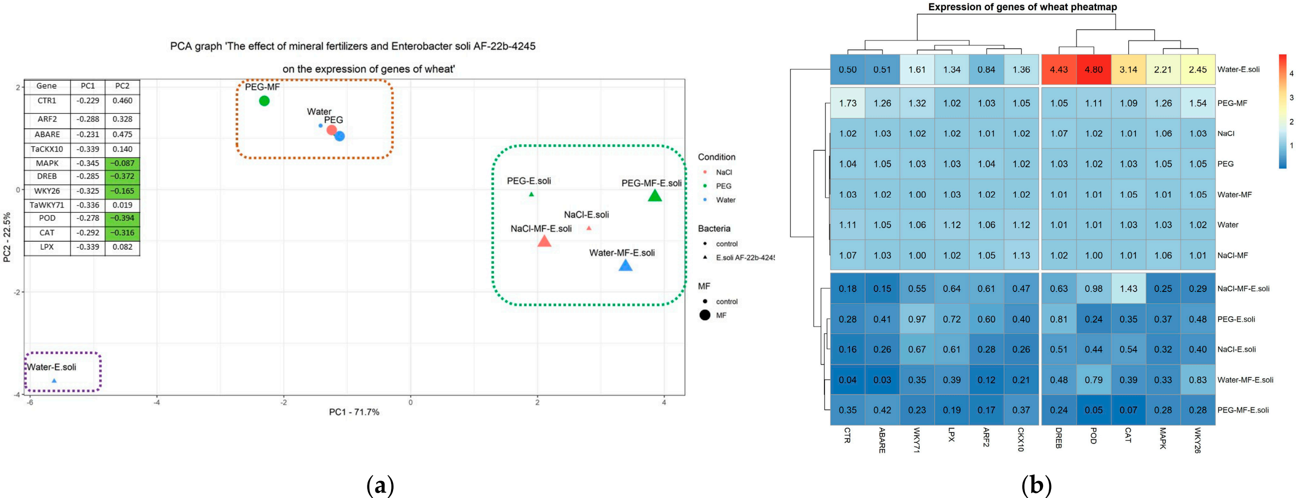


Figure 7. Analysis of expression levels of wheat genes: (**a**) PCA on data of expression levels of wheat genes under different conditions (**b**) Heatmap of expression levels of wheat genes under different conditions.

Based on the obtained results, it can be seen that the addition of bacteria leads to the activation of genes involved in protection against peroxide radicals (*CAT, POD*), as well as signal transduction proteins involved in the implementation of anti-stress responses (*DREB*, *WKY26*) and some hormonal signals (*MAPK*). It means that *E. soli strain AF-22b-*

4245 activates the anti-stress protection of the plant. Probably, its positive effect may be associated with this, especially in stressful conditions, since the plant seems to be already metabolically preparing for stress and can physiologically compensate for it from the very beginning. Interestingly, in all conditions of bacterial addition (minerals, salt, drought), on the contrary, there is a decrease in the expression of all stress response genes, as if in these conditions the addition of bacteria dampens the development of stress reactions. We can assume that under these conditions, the bacterium either stopped releasing some factors into the environment that caused the activation of the anti-stress response in plants, or on the contrary began to release substances that reduce the anti-stress response in plants.

3.3.3. Growing Wheat in Conditions of Lack of Nutrients

According to screening tests and the results of genome annotation, it was found that *E. soli strain AF-22b-4245* was able to solubilize phosphate, probably by secreting citric and fumaric acids. In addition, wheat showed increased growth in all tests, with and without stress. In order to assess the strength of the phosphate solubilization effect, as well as to identify whether the increased growth is caused by the ability of *E. soli strain AF-22b-4245* to supply nitrogen to plants, we modeled an experiment in which the control group of plants received a complete set of mineral fertilizers, and the comparison groups received everything except for one component: nitrogen, magnesium, and phosphorus. Previously, we found that when there was a lack of lighting, plants accumulated a deficiency of nitrogen, phosphorus, and magnesium (data have not been published), so the experiment was carried out with a 12 h illumination of plants. The result was evaluated by measuring the growth and biochemical characteristics (Table 6, Figure 8).

The idea of the experiment was to identify the group of plants that, according to the totality of all characteristics, is close to the conditions of full nutrition. The most convenient way to assess this is PCA analysis (Figure 9). According to its results, the cluster is clearly visualized (green outline): complete nutrition (NPMg), complete nutrition and bacterial suspension (NPMg-1156), and bacterial suspension with mineral fertilization without phosphorus (NMg-1156). In other words, the bacterial suspension of *E. soli strain AF-22b-4245* compensated for the lack of soluble phosphorus in the mineral fertilizer, probably by solubilizing insoluble forms in the soil. Separately, it is necessary to note that all plants receiving nitrogen were grouped into one large cluster (red contour) against those who did not receive it (blue contour). From Table 6, it is easy to see that plants receiving nitrogen showed the highest growth rates.

Table 6. Growth and biochemical characteristics of wheat when grown under conditions of lack of nutrients.

Nutrition	Bacteria	Plant Height (cm)	Leaf Fresh Weight (g plant^{-1})	Root Fresh Weight (g plant^{-1})	Leaf Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	Chl A/Chl B
Water	No	18.9 ± 1.42	0.35 ± 0.02	0.65 ± 0.12	0.17 ± 0.03	0.18 ± 0.02	1.83 ± 0.12
Water	Yes	18.8 ± 1.35	0.44 ± 0.03	0.88 ± 0.05	0.19 ± 0.05	0.24 ± 0.01	1.41 ± 0.30
def N	No	23.6 ± 0.6	0.53 ± 0.09	0.72 ± 0.05	0.18 ± 0.04	0.23 ± 0.04	1.90 ±0.03
def N	Yes	22.9 ± 1.4	0.55 ± 0.06	0.99 ± 0.13	0.20 ± 0.03	0.28 ± 0.04	1.96 ± 0.10
def Mg	No	32.1 ± 5.1	1.17 ± 0.04	1.30 ± 0.22	0.38 ± 0.06	0.42 ± 0.09	1.84 ± 0.09
def Mg	Yes	40.3 ± 0.3	1.48 ± 0.06	1.57 ± 0.12	0.58 ± 0.04	0.57 ± 0.07	1.68 ± 0.09
def P	No	32.8 ± 2.2	1.13 ± 0.08	1.36 ± 0.09	0.38 ± 0.12	0.46 ± 0.06	1.91 ± 0.02
def P	Yes	46.2 ± 2.8	1.59 ± 0.23	1.65 ± 0.26	0.56 ± 0.09	0.64 ± 0.06	1.75 ± 0.06
NPMg	No	38.9 ± 2.9	2.05 ± 0.24	1.82 ± 0.21	0.69 ± 0.01	0.68 ± 0.08	1.29 ± 0.20
NPMg	Yes	48.1 ± 8.2	2.47 ± 0.10	1.85 ± 0.35	1.04 ± 0.18	0.67 ± 0.06	1.07 ± 0.31

Def N—deficit nitrogen; def Mg—deficit magnesium; def P—deficit calcium phosphate, NPMg—all mineral fertilization added; water—only water added.

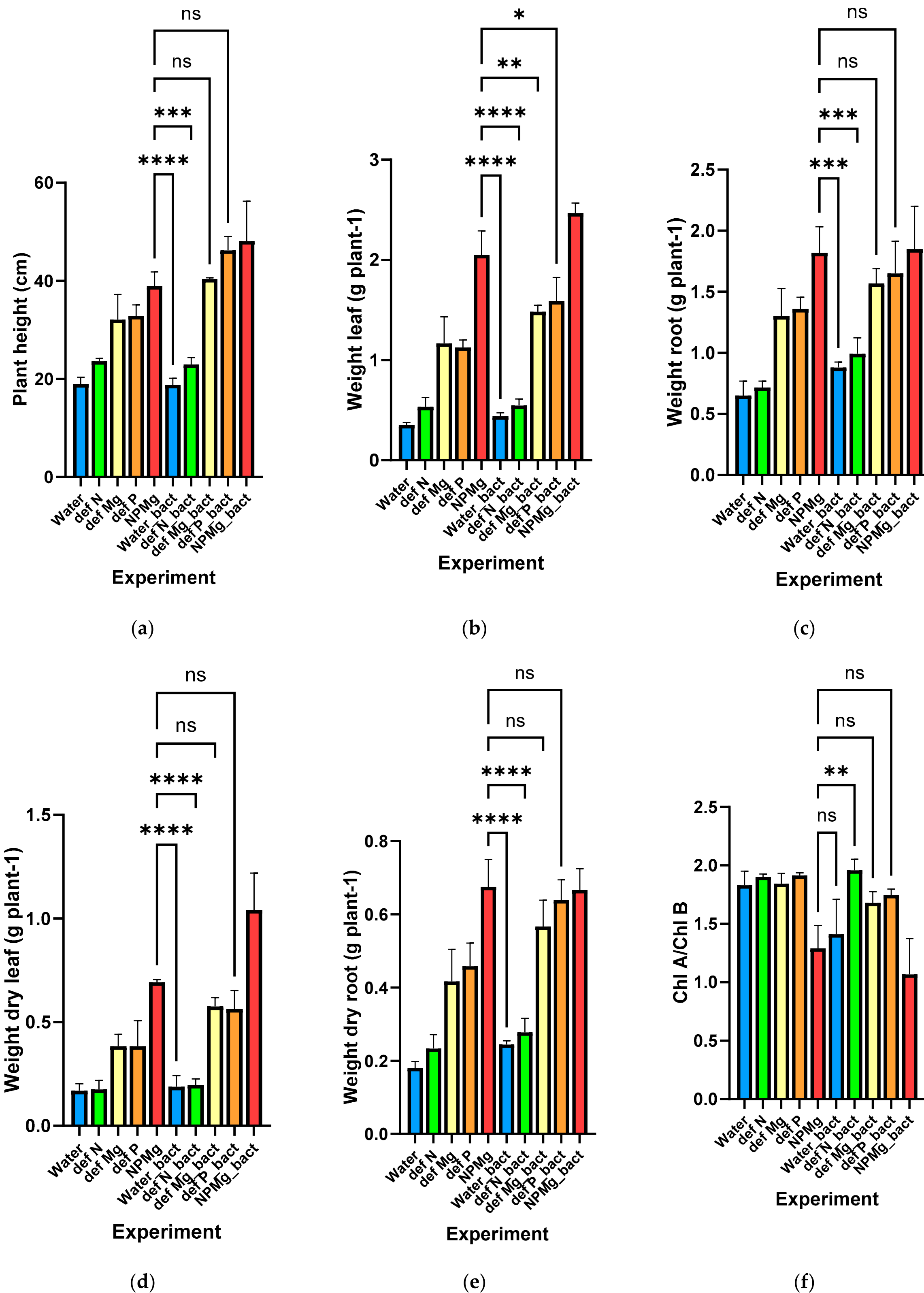
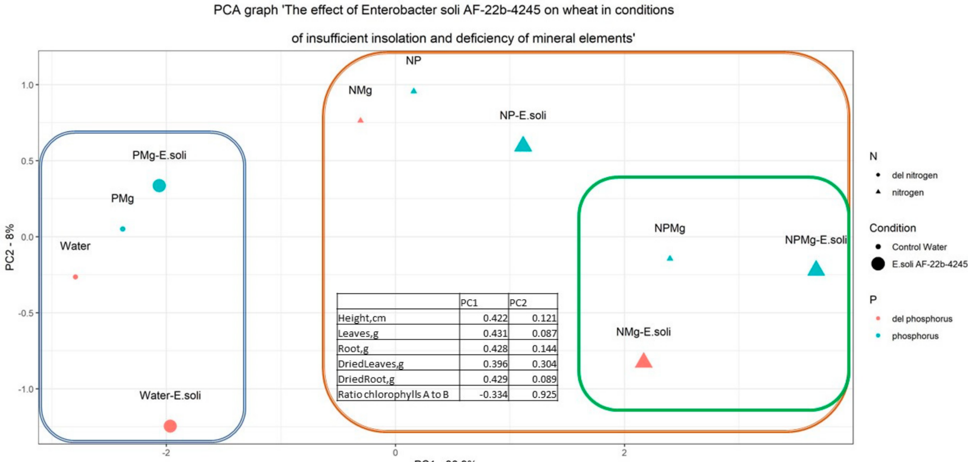


Figure 8. Diagrams of economic indicators of plants after treatment with bacteria and mineral fertilizer: (**a**) height of the plant, (**b**) the weight of the aboveground part of the plant, (**c**) the weight of the underground part of the plant, (**d**) the dry weight of the aboveground part of the plant, (**e**) the dry weight of the underground part of the plant, (**f**) ratio of chlorophyll A to B. Statistically significant values are marked by asterisk: *—level $p < 0.05$, **—level $p < 0.01$, ***—level $p < 0.001$, ****—level $p < 0.0001$; ns—not significant.



	PC1	PC2
Height,cm	0.422	0.121
Leaves,g	0.431	0.087
Root,g	0.428	0.144
DriedLeaves,g	0.396	0.304
DriedRoot,g	0.429	0.089
Ratio chlorophylls A to B	-0.334	0.925

Figure 9. PCA analysis of nutrient deficiency compensation due to PGBP properties of *Enterobacter soli* AF-22b-4245.

4. Discussion

In this paper, we conducted a study of the *Enterobacter soli strain AF-22b-4245* for the presence of PGPB properties, genes responsible for these properties, and the effect of this strain on wheat plants.

4.1. The PGPB Properties and Ability of the Strain to Grow Under Stressful Conditions

According to the results of primary screening, the ability of *E. soli strain AF-22b-4245* to produce phytohormones (IAA, gibberellins), ammonium, and solubilize phosphate was shown. Tests on the ability to survive in an environment with heavy metals showed that the *E. soli strain AF-22b-4245* was able to grow in the presence of copper and lead.

In addition, primary screening revealed the ability of the *E. soli strain AF-22b-4245* to grow in conditions of drought and elevated salt levels. It was found that the *E. soli strain AF-22b-4245* tolerated drought conditions well, was able to survive at a substrate-metric potential of -0.30 MPa (PEG concentration of 15%), and tolerated salinity well (up to 5% of NaCl). Soil salinity is a serious obstacle for agriculture. Salt stress adversely affects the formation of seedlings, plant growth, root structure, and nutrient absorption [4]. An excess of Na+ in the soil prevents plants from assimilating K+ and increases the accumulation of active oxygen, which leads to oxidative damage [62].

It was shown that under salt stress, salt-resistant strains (*H. variabilis HT1* and *P. rifietoensis RT4*) demonstrated higher biofilm formation [63], which suggests that biofilm formation in this case may be a protective response mechanism to adverse external conditions. It is likely that the ability of the *E. soli strain AF-22b-4245* to survive in conditions of increased salinity, up to 5% NaCl (Table 1), shown in screening tests, is a consequence of biofilm formation. A similar result was observed when investigating the mechanism of salt tolerance in the rhizosphere bacterium *Rahnella aquatilis strain JZ-GX1*. This strain enhances salt tolerance by promoting the production of extracellular polysaccharides and is able to secrete up to 60.6 milligrams per liter (mg/L) of these polysaccharides at a high salt concentration of 9%. Additionally, the accumulation of trehalose, a compatible soluble substance, has also been demonstrated for this strain, which could explain its superior tolerance to high salt levels [64].

PGPB may also enhance plant growth in stress conditions through indirect mechanisms, including nitrogen fixation from the atmosphere, hydrolysis of inorganic phosphates, chelation of iron, and production of plant hormones. *E. soli strain AF-22b-4245*, according to the screening, produces IAA in an amount of 79.74 ± 17.54 µg/mL. High levels of IAA

synthesis have previously been reported for several other representatives of the genus *Enterobacter*. The *Enterobacter sp. SE992 strain* produces IAA through the tryptophan-dependent pathway, with the highest level observed when 1% tryptophan (200 μg/mL) is added to the growth medium, while lower levels of IAA are detected in media lacking tryptophan [65]. The *Enterobacter sp. PAB19 strain* produces indole-acetic acid at a concentration of 176.2 ± 5.6 μg/mL [66], while for *Enterobacter cloacae* (Accession No. MG00145), IAA production has been reported up to 17.934 μg/mL [67]. Nevertheless, there exist representatives of the family *Enterobacteraceae* with valuable agronomic properties but with lower IAA biosynthesis. *Enterobacter cloacal PS14*, capable of combating potato wilt disease, named *Ralstonia solanacearum (Smith)*, produce 0.133 micrograms per milliliter (μg/mL) of IAA [68]. It is worth noting that *Enterobacter* strains exhibiting such a high level of phytohormone production have the ability to survive under unfavorable conditions. *Enterobacter sp. SE992* can survive in environments with 5% NaCl concentration, and *Enterobacter cloacae* (Access No. MG00145) can tolerate 9% NaCl and grow at 54 °C under osmotic stress conditions (40–45% polyethylene glycol 6000 in the medium). The *PAB19* strain exhibited exceptional tolerance to high levels of drought stress (18% PEG-6000).

4.2. Analysis of Genes Involved in the Development of Traits That Contribute to PGPB Properties

In this paper, we propose to annotate the genome using a list of genes that are responsible for the presence of traits useful for agriculture. Based on the presence of these genes in the genome of the studied microorganism, and in most cases on the functional group of genes, it is possible to judge the presence of certain properties that cannot be unambiguously determined by screening tests.

4.2.1. Biofilm Formation

Despite the fact that the screening test did not reveal the ability of *E. soli strain AF-22b-4245* to form a biofilm, according to the gene representation based on the results of whole-genome sequencing, the strain most likely has the ability to form it. In the recent work, Md. Manjurul Haque et al. also experimentally showed that another strain of *Enterobacter soli ES53G* formed a biofilm. Biofilm formation is considered a useful quality for PGPB, as it contributes to their survival in conditions of drought, salinity, high temperatures, and heavy metal pollution [58].

The biofilm formation contributes to the survival of not only the microorganism forming it, but also has a positive effect on plants. The following mechanisms suggest how biofilm formation by microorganisms promotes plant growth in saline soils: (1) biofilm acts as a physical barrier, by enveloping the roots, thereby preventing direct contact with salt; (2) a moisture-retaining layer is created around the seeds, which ensures their survival during germination [60]. Due to the increased survival rate, PGPB fertilizers capable of forming biofilms are more practical and stable.

4.2.2. Phytohormone Production

As part of the screening for *E. coli strain AF-22b-4245*, the production of SA was shown at the level of 0.55 ± 0.40 μg/mL. Relative to other representatives of *Enterobacter*, these are low indicators. The *E. cloacae PS14* strain produces SA 1.488 μg/mL [68], and *Enterobacter sp. PAB19*—42.5 ± 3.0 μg/mL [66]. Perhaps the lowest production level revealed in the screening is due to the fact that the genome analysis of the *E. soli strain AF-22b-4245* lacks a biosynthetic cluster of genes in its genome contains both isochorismate synthetase and isochorismate lyase. Most likely, the screening method detects not only salicylic acid, but also its metastable precursor isochorismate [49].

4.2.3. Nitrogen Fixation

The ability of a microorganism to fix nitrogen has been confirmed in laboratory tests using several methods. The first method, which is used as a preliminary screening test, evaluates the capacity of microorganisms to grow in a nitrogen-free bromothymol blue semi-solid medium [69]. The second method, known as the "acetylene method", assesses nitrogenase activity based on the reduction in acetylene by nitrogenase and separation of acetylene and ethylene through gas chromatography [70]. The third method involves detecting the presence of the *nifH* gene using PCR with degenerate primers [71].

In our research, we found that the strain *E. coli AF-22b-4245* isolated on a nitrogen-free medium can grow on this medium, but it does not possess the nitrogen fixation genes previously described in the literature [38]. In the work by P. Santos, a minimum set of genes has been identified that allow the microorganism to fix atmospheric nitrogen, namely *nifHDKENB* [72]. This set of genes serves as the primary criterion for identifying diazotrophic microorganisms through bioinformatics methods.

The ability of four strains of microorganisms to grow in nitrogen-free environments using alternative sources of carbon, nitrogen, and energy has been previously described. These strains include *Pseudomonas putida strain* 1290 [73], *Pseudomonas phytofirmans PsJN* [74], *Acinetobacter baumannii ATCC 19606* [75], and *Enterobacter soli LF7* [76]. Based on experimental evidence, it is hypothesized that these bacteria can grow due to the presence of IAA, which is catabolized by a cluster of genes (*iacR-iacHABICDEFG*) in these microorganisms. All of these bacteria have been isolated from plant sources that contain IAA at concentrations sufficient to support bacterial growth [76]. However, despite being closely related to *E. soli strain AF-22b-4245*, *Enterobacter soli LF7*, we did not find a set of iac genes in *E. soli AF-22b-4245* during our study. It is likely that these microorganisms have alternative mechanisms for survival in minimal environments that involve catabolism of metabolites such as IAA. Therefore, screening tests such as growth on nitrogen-free media and polymerase chain reaction (PCR) using degenerate primers may not be conclusive in determining whether a microorganism has the ability to fix nitrogen.

4.3. The Influence E. soli AF-22b-4245 Strain on Wheat Growth and Gene Expression

4.3.1. Effect on Plant Growth

A combined analysis of screening tests and genome annotations of the *E. soli strain AF-22b-4245* indicated that it was potentially capable of solubilizing phosphate and producing phytohormones, while being in conditions of drought or salt stress. Stress from drought and salinization, individually or in combination, reduce the productivity of one of the vital food crops—wheat [77]; therefore, this crop was chosen for greenhouse experiments. Greenhouse experiments were carried out according to the scheme from simple to complex as follows. First, the effect of the *E. soli strain AF-22b-4245* was evaluated under standard conditions in combination with and without mineral fertilization. The greatest statistically significant increase in indicators (plant height, weight of the aboveground part, weight of roots, and the aboveground part after drying) was shown with the combined use of mineral fertilization and the introduction of bacterial suspension under the root. This suggests that the *E. soli strain AF-22b-4245* increases the efficiency of mineral fertilizers.

This is consistent with previously obtained data, which suggests that the application of mineral fertilizers may have a beneficial impact on the effectiveness of microbial fertilizers. It is likely that this is due to an increase in the number of bacteria that promote plant growth or enhance plant diversity [78,79].

We also attempted to determine which specific constituents in the formulation of mineral fertilizers are essential for achieving their beneficial effects. We conducted an experiment involving the addition of mineral fertilizer without nitrogen, phosphorus,

or magnesium. The findings indicate that nitrogen is the most crucial component in mineral fertilization. Interestingly, the principal component analysis (PCA) of the results demonstrated that the presence of the E. soli strain AF-22b-4245 compensates for the absence of soluble phosphorus in plants, which aligns with screening tests and genome annotations.

4.3.2. Gene Expression

We estimated the expression levels of wheat genes that, according to the literature, are involved in the stress response, such as the genes of proteins that perceive the stress signal (*ABARE*, *CTR1*), signal transduction proteins and transcription factors *(DREB, MAPK, WKY26, WKY71)*, proteins regulating the biosynthesis of osmolytes and other secondary metabolites (*ARF2, CKX10*), as well as proteins involved in the restoration of redox homeostasis in cells (*CAT, LPX, POD*) (Figure 3). It was found that the addition of *Enterobacter soli AF-22b-4245* suspension activates the anti-stress protection of the plant. Probably, its positive effect may be associated with this, especially in stressful conditions, since the plant seems to be already metabolically preparing for stress and can physiologically compensate for it from the very beginning. Interestingly, under all conditions of bacterial presence (mineral, salt, drought), there is, on the contrary, a decrease in the expression of all genes that respond to stress, as if, in these conditions, the presence of bacteria inhibits the development of stress responses. We can assume that under these conditions, the bacterium either stopped releasing some factors into the environment that caused the activation of the anti-stress response in plants or on the contrary began to release substances that reduce the anti-stress response in plants.

5. Conclusions

The *E. soli strain AF-22b-4245* showed a unique combination of properties in phenotypic tests. It tolerates salinity (up to 5% NaCl), drought (up to PEG 15%), copper, and lead. It is a good producer of phytohormones (IAA, gibberellins), ammonium, and siderophores. In addition, high rates of phosphate solubilization were recorded.

Genome analysis has demonstrated the presence of genes of biofilm formation, siderophore production (enterobactin synthesis genes), production of IAA, 1-aminoacyclopropane-1-carboxylate gene (*accD*), citric acid (*citC*) and fumaric acid (*dcuS*) secretion genes, *arsC* (arsenic reduction), and *chrR* (chromium reduction) reductase genes, genes for response to oxidative stress (*soxR, soxS, CatB, sodA*, and *sodB*).

When modeling the conditions of drought and salinity, we showed that the effect of the combined use of mineral fertilizers and bacterial suspension was maximal, which indicates that the positive effect of the *E. soli strain AF-22b-4245* on wheat growth and development persists even under stress conditions. Several factors in the studied bacterium can contribute to this: biofilm formation, active synthesis of indoleacetic acid, and increased phosphorus availability. Our studies on the evaluation of the gene expression of the stress response suggest that the primary effect of introducing bacteria may be due to the activation of stress response proteins, while this mechanism does not seem to be involved directly during stress.

In total, the results of our study show that the *Enterobacter soli strain AF-22b-4245* is promising for use in arid or saline soils in order to increase the efficiency of mineral fertilizers, as well as to increase the availability of soil phosphates by solubilizing them.

The limitation of our study is that we did not carry out a preliminary selection of wheat varieties resistant to salt stress and drought. Recently, a paper was published in which a meta-analysis of QTL was carried out, identifying the regions of the genome according to which wheat varieties should be selected, by adapting it to combined stresses. For example, the *ERD15* gene differentially expressed in different wheat genotypes under conditions

of combined stress, largely regulates water balance, photosynthetic activity, antioxidant activity, and ionic homeostasis [80]. Probably, a combined approach, including selection of varieties, application of mineral fertilizers, and treatment with suspensions of target bacterial strains, will give the maximum effect.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres16020034/s1; Table S1: PGBP genes of *E. soli strain AF-22b-4245* based on genome annotation. Table S2: ORF of *E. soli strain AF-22b-4245* predicted by GeneMark.

Author Contributions: Conceptualization, E.N.V.; evaluation of the properties of bacteria useful for plants, O.V.M. and I.N.T.; analysis of genome-wide sequences and statistical data analysis, E.A.S.; working with plants, E.V.C.; RNA isolation and analysis of gene expression, I.V.K. and O.V.M. All authors have read and agreed to the published version of the manuscript.

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