International Journal of
Plant Biology

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Plant Growth-Promoting and Biocontrol Characteristics of Four Bacillus Strains and Evaluation of Their Effects on Wheat (*Tr. aestivum* L.)

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Abstract: The present study investigated developing biological control agents against plant pathogens as an alternative to pesticides. The plant growth-promoting (PGP) and biocontrol potential of bacteria from the *Bacillus* genus is due to their ability to produce proteolytic and amylolytic enzymes, assist in the solubilization of phosphorus and zinc, and the production of siderophores. Cell culture and cell-free supernatant were used to investigate the antimicrobial activity of different *Bacillus* strains against the phytopathogenic fungus *Fusarium graminearum* in vitro. *Fusarium graminearum* is a fungus that causes plant disease, particularly in cereals like wheat and barley. As a result, significant suppression of the growth and development of this plant pathogen was observed. Plant growth-promoting activity manifested when the bacteria were applied alone and in combination. A single strain and combinations of two, three, and four strains of *Bacillus* were tested for their antimicrobial effects against *Fusarium graminearum*. The fluorescence spectroscopy results proved that the combination of *Bacillus subtilis*, *Bacillus circulans*, *Bacillus megaterium*, and *Bacillus licheniformis* showed the best stimulation of development, expressed as a comparative evaluation of the yield compared to the untreated control variant. The four strains showed their potential application as a biocontrol agent against *Fusarium graminearum*. The four *Bacillus* strains also can promote plant growth by affecting nutrition, root structure, and plant health, and they have the capacity to dissolve phosphates and zinc.

Keywords: *Bacillus* spp.; siderophores; antifungal activity; *Fusarium graminearum*; wheat; mobile fluorescence spectroscopy

Academic Editor: Adriano Sofo

Received: 24 November 2024
Revised: 15 December 2024
Accepted: 17 December 2024
Published: 27 December 2024

Citation: Petkova, M.; Marcheva, M.; Petrova, A.-L.; Slavova, V.; Shilev, S. Plant Growth-Promoting and Biocontrol Characteristics of Four Bacillus Strains and Evaluation of Their Effects on Wheat (*Tr. aestivum* L.). *Int. J. Plant Biol.* **2025**, *16*, 1. https://doi.org/10.3390/ijpb16010001

1. Introduction

The development of *Fusarium graminearum* on wheat has led to substantial economic losses and a decrease in the safety of cereal production. Chemicals used against this pathogen affect human health via pesticide residues on emergence and promote resistance to fungicides. Sustainable agriculture is a new approach to growing crops, relying heavily on biofertilizers that mimic natural ecological processes for plants' nutrition systems [1]. Proper fertilization with a balanced chemical, organic, and biofertilizer composition is the

key to success in increasing production. The use of plant growth-promoting rhizobacteria (PGPR) as a biofertilizer was reported by Gardener (2009) and Miljaković (2020) [2,3]. Rhizobacteria allow colonizing the rhizosphere and supplying primary nutrients, mainly nitrogen (N) fixation, phosphorus and zinc solubilization, and siderophores production (for iron chelation). Other methods include the production of phytohormones such as indole acetic acid (IAA), gibberellic acid (GA), etc., the production of ammonia (as a source of N), and providing tolerance against adverse conditions (high salinity and drought). Selected beneficial bacteria naturally associated with plants are used as biological agents against phytopathogens [4].

These bacteria are aerobic, with relatively high growth rates and low nutrient requirements, and they can grow on various nutrient sources. They usually produce a range of enzymes (cellulases, amylases, and proteases), degrading various substrates derived from naturally abundant sources such as lignocellulose, starch, proteins, hydrocarbons, and biofuels [5]. An essential advantage of *Bacillus* spp. is their ability to form endospores, which makes them exceptionally resistant to abiotic stress such as heat and drought. Rhizobacteria improve plant growth through nitrogen fixation, phosphate and zinc solubilization, phytohormone production, and the mitigation of the effects of some abiotic factors. Their ability to colonize roots contributes to the secretion of antimicrobial compounds and stimulators of plant resistance. This metabolic secretion enables the fight against a diverse set of bacteria, viruses, and fungal pathogens with different life cycles and modes of virulence. *Bacillus* spp. can inhibit the growth and development of Gram-negative bacteria and reduce the diseases caused by these pathogens, as one strain can act against several bacterial pathogens. *Fusarium graminearum* is one of the most important wheat pathogens that cause head-blighting in Bulgaria. The approaches established for the control of *Fusarium* spp. infections on cereals are mainly fungicide application, agrotechnical practices, and use-resistant cultivars [6–8]. *B. subtilis*, *B. megaterium*, *B. licheniformis*, and *B. circulans*, commonly found in soils, are considered safe for use in the environment and with mammals [7]. They produce antagonistic substances against several fungal and bacterial pathogens [8] and can be retained in the plant to provide higher protection [9]. Their antagonistic activity is often associated with producing secondary metabolites with antibiotic properties [10], mostly characterized as dipeptides or cyclic peptides of low molecular weight [11]. The metabolites produced by *Bacillus* spp. may also affect microflora in the rhizosphere, offering an unfavorable environment to pathogens or activating host defense responses [12–14]. Ashraf et al. in 2004 found that the *Bacillus* spp. strain SCH1 binds to Na^+ ions, reducing its concentration and helping plant growth by producing exopolysaccharides [15]. Moreover, they are among the most widespread bacteria that have been shown to colonize plants endophytically, play an essential role in the biocontrol of plant pathogens, and enhance systemic resistance to abiotic stress [16]. The biological control of plant diseases involving microorganisms or compounds of biological origin is considered one of the most promising alternatives to chemical pesticides. Because it often affects several modes of action to limit the growth of microbial pathogens, biocontrol is environmentally friendly. The mechanisms of action of biological agents include competition for space and nutrients, parasitism, antibiosis, and stimulating host plant defenses. *Bacillus* species are widely distributed in the soil, where they help recycle carbon and nitrogen compounds through the production and secretion of macromolecular hydrolases, proteases, amylases, and cellulases. Those species produce a range of secondary metabolites, including indole-3-acetic acid, terpenes and siderophores, and ribosomally and nonribosomally synthesized peptides. Lipopeptides synthesized by bacteria of the genus *Bacillus* have a cyclic structure formed by 7–10 amino acids and beta-hydroxy fatty acid. These lipopeptides exhibit anti-inflammatory, antiviral, and antifungal properties and are involved in forming biofilms [17]. The *Bacillus* lipopeptides are usually

divided into three groups, the surfactin, iturin, and fengycin families, based on their specific peptide chains and fatty acid structures [18,19]. Following recent studies, the antifungal activity of bacterial lipopeptides was sustained on terminal membrane pore formation and at a low concentration to induce apoptosis [18,19]. They are classified into three families: surfactin, iturin, and fengycin [20–22]. Iturin and fengycin have been reported chiefly with antifungal activity [23,24], while surfactin exhibited antibacterial, antifungal, and antiviral activity, according to Shaligram and Singhal, 2010 [25].

Siderophores are small, low-weight (between 500 and 1500 daltons) iron-chelating compounds with high affinity that are secreted by microorganisms [26]. Siderophore production is associated with forming soluble Fe^{3+} complexes, which are involved in active membrane transport and can be taken up by plant cells [27]. In response to iron limitation in their environment, the microbes' genes involved in the production and uptake of siderophores are depressed, resulting in the production of siderophores and appropriate proteins for uptake [28]. Fe^{2+}-dependent repressors in bacteria bind to genes involved in siderophore production at high intracellular iron concentrations. At low concentrations, Fe^{2+} dissociates from the repressor, which dissociates, leading to gene transcription [29]. The synthesis of siderophores by microorganisms in the rhizosphere also leads to the difficulty of iron access to harmful microflora, which is reported as a significant PGP feature [30,31].

The present research aims to develop a novel composition for producing a biological preparation suitable for wide application in agriculture. This preparation is designed to enhance plant growth and development by facilitating the release of growth hormones, which play a critical role in improving crop yield and resilience. Additionally, the preparation will exhibit phosphorus- and zinc-solubilizing capabilities, thereby increasing the bioavailability of these essential nutrients to plants. Furthermore, by producing antimicrobial peptides, this biological preparation will effectively protect wheat plants against economically significant diseases, contributing to sustainable agricultural practices and improved crop health.

2. Materials and Methods

2.1. Bacterial Strains

B. subtilis NBIMCC 2353, *B. megaterium* NBIMCC 8947, and *B. circulans* NBIMCC 1042 were purchased by the Bulgarian National Collection for Microorganisms and Cell Cultures and provided by LB-LACT BAS, Plovdiv, Bulgaria. *B. licheniformis* was isolated from soil and identified according to Petkova and Dimova, 2024 [32]. The 16S rRNA gene was sequenced with universal primers (27F and 1492R) [33] at Microsynth (Balgach, Switzerland) and the obtained sequences were compared with world databases (https://blast.ncbi.nlm.nih.gov/ and https://www.ezbiocloud.net/; accessed on 16 May 2024). The accession in the NCBI Genbank is under submission (Accession: PP797577.1, SUM14450584, 8 June 2024). A previous study of Gito et al. (2000) demonstrates that sequencing using these primers is sufficient for identifying relevant bacterial pathogens and assessing microbial diversity in environmental samples [34].

2.2. Amylolytic Activity of Tested Bacillus Strains

The isolated four different strains of the genus *Bacillus* were qualitatively and quantitatively evaluated for the presence of amylolytic activity by the agar diffusion method [35]. Starch agar plate (hard), g/L: wheat starch—10; peptone—5; yeast extract—5; $MgSO_4 \cdot 7H_2O$—0.25; $FeSO_4 \cdot 7H_2O$—0.01; agar—15; pH 6.8 ± 0.2. In the agar-diffusion method, the nutrient medium was poured in 15 cm^3 and, after solidification in the petri dish, four wells with a diameter of 9 mm were made. Two samples were prepared for each strain: CM-culture

medium from a 24 h suspension of the strain and CFS-cell-free supernatant obtained by centrifugation of culture fluid at 5000× *g* (Universal 320 R, Hettich, Tuttlingen, Germany). The bacterial suspension or CFS from each isolate was instilled in 100 µL. After incubation at 28 °C for 48 h, the Petri dishes were treated with Lugol's solution (3% KI) to form a blue-colored starch–iodine complex. A halo area was observed around the amylolytic bacterial colonies. The result was reported by measuring the diameter of the formed halo zone in mm.

2.3. Proteolytic Activity of Bacillus Strains

The proteolytic activity of the tested bacteria was assessed by inoculation on milk agar, according to Nabrdalik, 2010 [36], containing casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skimmed milk powder 2.5%, and agar 1.5%. Bacterial isolates were pre-activated in Tryptic soy agar (TSA) (Merck KGaA, Darmstadt, Germany). The proteolytic activity of each strain was investigated as culture fluid—CM obtained from 24 h culture and cell-free supernatant—CFS obtained by centrifugation of culture fluid. Wells with a diameter of 9 mm were formed on agar medium with added skimmed milk, in which 100 µL of the previously prepared cultures and cell-free extracts were placed. The samples were cultured at 28 °C and the result was that the diameter of the proteolytic zones measured in mm was reported after 24, 48, and 72 h.

2.4. Quantification of the Synthesized Secondary Metabolites with Phytohormonal Activity

The synthesis of microbial phytohormones by soil microorganisms is associated with signal changes in root and plant growth stimulation. A phytohormone in the microbial culture supernatant cannot prove the molecule's functional role in its interaction with the plant. Several studies have observed a correlation between plant growth and hormone concentration measured in culture medium or colonized plant tissues in experiments and in situ [37].

2.4.1. Quantification of Indole-3-Acetic Acid Using the Salkowski Reagent

For the quantification of indole-3-acetic acid (IAA) produced, bacterial isolates were grown in a tube in medium with or without 0.1% (*w*/*v*) L-tryptophan (L-Trp) and incubated in the dark at 30 °C and for 5 days, as was published previously by Petkova et al. 2022 [38]. One milliliter of the cells was pelleted by centrifugation at 3000× *g* for 5 min and 0.5 mL of the supernatant was mixed with 0.5 mL of Salkowski's reagent (2 mL of 0.5 M iron (III) chloride and 98 mL of 35% perchloric acid) [39]. After 30 min, color development (red) was quantified using a spectrophotometer (Unico 1200-Spectrophotometer, UNICO, Dayton, NJ, USA) at 530 nm. A calibration curve using pure indole-3-acetic acid was established to calculate IAA concentration (Supplementary Figure S1). The IAA production of each bacterial isolate was determined by inoculating L-tryptophan medium containing 0.1% (*w*/*v*) L-tryptophan and incubating in the dark at 28 °C. After incubation, the IAA produced was quantified spectrophotometrically.

2.4.2. Screening for Dissolution of Inorganic Phosphates

Screening for dissolution of inorganic phosphates was performed using Pikovskaya's medium (PVK), as published by Petkova et al. 2022 [38], containing glucose, 10 g; $Ca(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4 \cdot H_2O$, 0.002 g; and $FeSO_4 \cdot 7H_2O$, 0.002 g.

2.4.3. ZnO Dissolution Screening

Isolates were tested for zinc solubilization ability using a modified ZnO medium [40]. The medium included 10.0 g glucose, 1.0 g ammonium sulfate, 0.2 g potassium chloride,

0.2 g dipotassium hydrogen phosphate, 0.1 g magnesium sulfate, and 0.1% insoluble zinc from a ZnO source in 1000 mL distilled water at pH 7.0. From a 24 h culture of isolates, 100 µL was dropped onto 10 mm petri wells containing said medium and incubated at 28 °C for 72 h. Colony diameter and halo areas counted were recorded. The solubility index was calculated using the clear zone diameter/colony diameter formula [41].

2.5. Screening Bacillus spp. for Genes Encoding Lipopeptides Iturin, Surfactin, and Fengycin

The isolation of DNA from the 24 h biomass of the cultures in LB broth (Carl Roth GmbH Co., KG, Karlsruhe, Germany), done with the Gene JetTM Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltman, MA, USA), was performed according to the manufacturer's instructions. PCR amplification was performed with DNA from *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus circulans*, and *Bacillus megaterium* and specific primers for detection of *ituC* (iturin A synthetase C), itrC_Fw primer 5′-GGCTGCTGCAGATGCTTTAT-3′ and itrC_Rv primer 5′-TCGCAGATAATCGCAGTGAG-3′, *srfAA* (surfactin) srfAA_Fw primer 5′-TCGGGACAGGAAGACATCAT-3′ and srfAA_Rv 5′-CCACTCAAACGGATAAT CCTGA-3′, and *fenD* (fengycin synthetase) fenD_Fw primer 5′-GGCCCGTTCTCTAAATCCAT and fend_Rv primer 5′-GTCATGCTGACGAGAGCAAA-3′, according to the method of by Joshi and McSpadden, 2006 [42]. PCR was carried out in a total volume of 20 µL containing 2× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0 U of Taq DNA polymerase (Biorad, Hercules, CA, USA), 0.2 µM of each primer, and 50–70 ng of genomic DNA. The cycling conditions for the amplification of all targets were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min. A final extension step at 72 °C for 5 min was followed by holding at 4 °C. The amplicons were separated on 1% agarose gel carried out in 0.5× TBE buffer (45 mmol/L Trisborate and 1 mmol/L EDTA) for 60 min at 100 V using a VWR Mini Electrophoresis system (VWR, Darmstadt, Germany) and MiniBis Pro (DNR Bio-Imaging Systems, Modi'in-Maccabim-Re'ut, Israel) for gel visualization after staining with Safe View (NBS Biologicals, Huntingdon, England).

2.6. Antimicrobial Activity Against Fusarium graminearum

The bacterial strains were tested to determine their antifungal activities against *Fusarium graminearum* NBIMCC 2214. *F. graminearum* was cultivated at 25 °C on yeast extract peptone dextrose (YPD) agar (10 g/L yeast extract, 20 g/L glucose, and 20 g/L peptone, and 20 g/L agar) (Merck KGaA, Darmstadt, Germany) for 7 days. Then, the conidia were collected by washing with cold sterile distilled water and used to prepare an inoculum at a concentration of 1×10^6 spores/mL. Antifungal bacterial activity tests were performed using the co-cultivation of *F. graminearum* with the bacterial strains. As a control, only *F. graminearum* was inoculated. A zone of inhibition (clear zone) was observed between the bacterial streak and fungal growth, suggesting antifungal activity [7]. Quantification of the results was done by utilization of the following equation;

> Inhibition, % = Radial growth on control plate with growth of *F. graminearum* (mm)/Radial growth in dual culture plate (mm) × 100

The scale was as follows: strong inhibition: >50% inhibition, moderate inhibition: 30–50% inhibition, and weak inhibition: <30% inhibition [7].

2.7. Field Experiment

The common winter wheat variety Teres is an experimental crop with an extensive distribution in Bulgaria. The specific susceptibility of the Teres variety is not well-documented in current studies, but general findings indicate that Fusarium resistance traits can be

mapped through genome-wide association studies and are influenced by phenotypic and genetic factors such as plant height and flowering time.

The trial was set at the experimental field of the Agricultural University—Plovdiv with a randomized complete block design. The field is located at 42°08′44.3″ N 24°48′55.6″ E (Supplementary Figure S2). The soil is Mollic fulvisols—FAO, with 25–28 cm of horizon A, medium sandy-clay, 3.7% humus, and pH 7–7.2. Soil analyses showed a low content of mineral nitrogen (29–40 mg/kg), low to moderate mobile P_2O_5 (By Egner-Reem) 5.0–9.8 mg/100 g, and good content of mobile K_2O (in 2N KCl) 37–41 mg/100 g. Standard plots of 10 m^2 (7.7 × 1.4 m) were sown with plot seeder Wintersteiger AG on the 20th of October 2022 with 450 germinating seeds/m^2. The soil drench technique with tested microorganisms was applied alone or in double, triple, and quadruple combinations at 300 mL/dka concentrations. The variants of treatment with the studied *Bacillus* strains in triplicates are presented in Table 1. This was performed at the beginning of tillering (growth stage—GS21–22). Sixteen treatment variants of the wheat variety *Teres*, with or without applying *Bacillus* species (used individually or in combination), were monitored and evaluated in triplicate during the vegetation period. Common cultivation practices for the crop were used, but no fungicides were spread to allow for monitoring the effect of the four *Bacillus* spp. on the phytopathological status of the wheat. Harvest was done by plot harvester Wintersteiger AG at full maturity when the grain reached 13% RH. Grain yields are presented as t/ha. Plants grown in the field were monitored for attack by phytopathogens, but *F. graminearum* on wheat was not reported during the current growing season.

Table 1. Schema of treatment of plants with bacilli alone or in separate combinations.

№	Treatment Combination	Treatment with *Bacillus* Strains	№	Treatment Combination	Treatment with *Bacillus* Strains
1	Control	No microorganisms applied	24	13 (1, 3, 4)	*Bacillus subtilis* + *Bacillus circulans* + *Bacillus megaterium*
2	1	*Bacillus subtilis*	25	15 (1, 2, 3, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*
3	2	*Bacillus licheniformis*	26	12 (1, 2, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus megaterium*
4	3	*Bacillus circulans*	27	2	*Bacillus licheniformis*
5	4	*Bacillus megaterium*	28	14 (2, 3, 4)	*Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*
6	7 (1, 2)	*Bacillus subtilis* + *Bacillus licheniformis*	29	11 (1, 2, 3)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans*
7	5 (1, 3)	*Bacillus subtilis* + *Bacillus circulans*	30	6 (1, 4)	*Bacillus subtilis* + *Bacillus megaterium*
8	6 (1, 4)	*Bacillus subtilis* + *Bacillus megaterium*	31	2	*Bacillus licheniformis*
9	8 (2, 3)	*Bacillus licheniformis* + *Bacillus circulans*	32	4	*Bacillus megaterium*
10	9 (2, 4)	*Bacillus licheniformis* + *Bacillus megaterium*	33	11 (1, 2, 3)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans*
11	10 (3, 4)	*Bacillus circulans* + *Bacillus megaterium*	34	10 (3, 4)	*Bacillus circulans* + *Bacillus megaterium*
12	11 (1, 2, 3)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans*	35	7 (1, 2)	*Bacillus subtilis* + *Bacillus licheniformis*

Table 1. *Cont.*

№	Treatment Combination	Treatment with *Bacillus* Strains	№	Treatment Combination	Treatment with *Bacillus* Strains
13	12 (1, 2, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus megaterium*	36	3	*Bacillus circulans*
14	13 (1, 3, 4)	*Bacillus subtilis* + *Bacillus circulans* + *Bacillus megaterium*	37	13 (1, 3, 4)	*Bacillus subtilis* + *Bacillus circulans* + *Bacillus megaterium*
15	14 (2, 3, 4)	*Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*	38	8 (2, 3)	*Bacillus licheniformis* + *Bacillus circulans*
16	15 (1, 2, 3, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*	39	12 (1, 2, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus megaterium*
17	7 (1, 2)	*Bacillus subtilis* + *Bacillus licheniformis*	40	1	*Bacillus subtilis*
18	3	*Bacillus circulans*	41	9 (2, 4)	*Bacillus licheniformis* + *Bacillus megaterium*
19	8 (2, 3)	*Bacillus licheniformis* + *Bacillus circulans*	42	5 (1, 3)	*Bacillus subtilis* + *Bacillus circulans*
20	1	*Bacillus subtilis*	43	4	*Bacillus megaterium*
21	5 (1, 3)	*Bacillus subtilis* + *Bacillus circulans*	44	14 (2, 3, 4)	*Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*
22	9 (2, 4)	*Bacillus licheniformis* + *Bacillus megaterium*	45	6 (1, 4)	*Bacillus subtilis* + *Bacillus megaterium*
23	10 (3, 4)	*Bacillus circulans* + *Bacillus megaterium*	46	15 (1, 2, 3, 4)	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*

2.8. Fluorescence Spectroscopy

The mobile fiber-optical spectral installation for studying fluorescence signals is explicitly designed to analyze plant biological samples rapidly. The experimental setup used by fluorescence spectroscopy includes the following components, as shown in Figure 1.

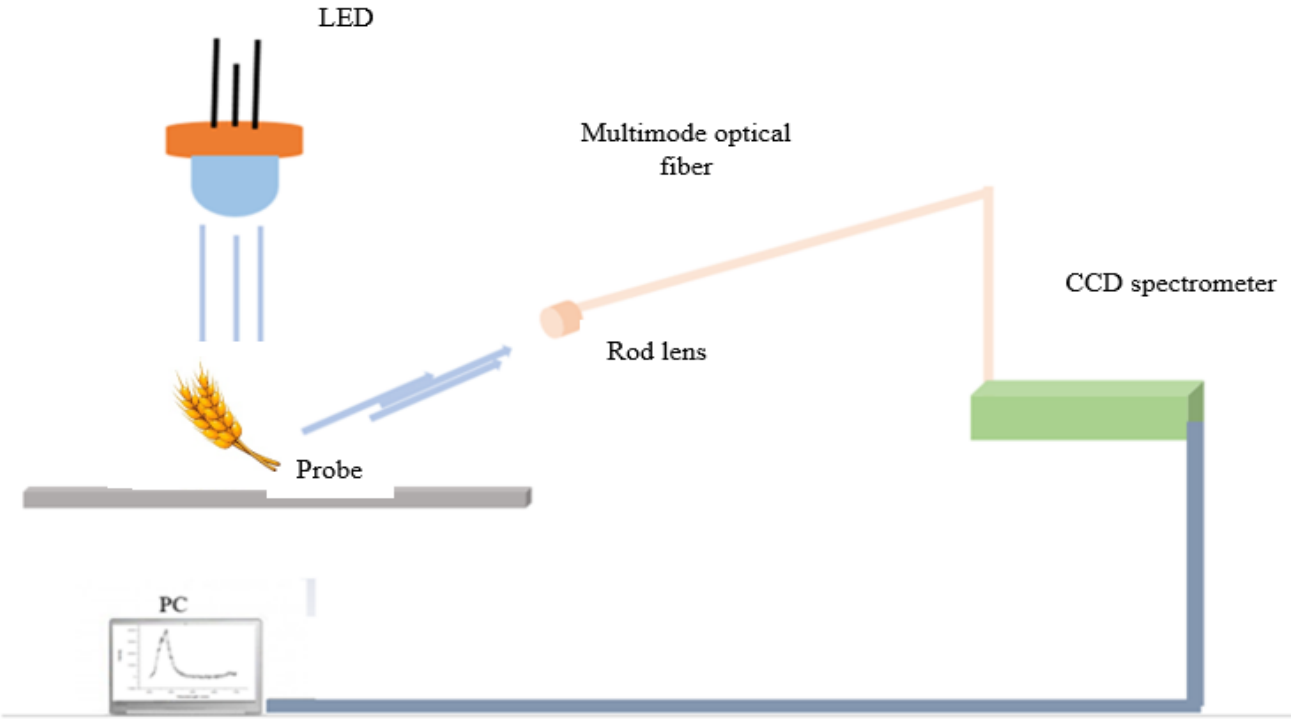

Figure 1. Mobile fiber-optic scheme for the measured fluorescence emission signals of individual wheat samples.

- A laser diode (LED) with an emission radiation of 245 nm with a supply voltage in the range of 3 V. It is enclosed in a hermetically sealed TO39-type metal case. The emitter current consumption is 0.02 A, its voltage drop is 1.9 to 2.4 V, and −6 V is the minimum emitter voltage value;
- The rod lens consists of two connected Schott and Corning lenses with anti-reflective coatings and different dispersion coefficients. It is of the achromatic doublet type. The chromatic aberration of one lens compensates for that of the other. This is due to the values of their radii. The forming optic has a diameter tolerance of −0.005 mm;
- The multimode optical fiber has a core diameter of 200 μm. The index of refraction is a step. It is FG200LEA;
- Four cm^2 is the area of the quartz glass. Its optical properties include being transparent to visible light and ultraviolet and infrared rays. For this reason, it is observed that no inhomogeneities scatter the light. Due to its purity, quartz glass's optical and thermal properties are superior to those of other types of glass. Quartz glass has a very low light absorption coefficient;
- The sensitivity of the CMOS detector is in the range of 200 nm to 1100 nm and $\delta\lambda = 5$ is its resolution. Unlike widely used sensors, the profile of the detector sensor projections used in this study along the X and Y axes is designed to generate very small amounts of data.
- The fiber optic setup used in this study has the following unique advantages:
- A rod lens was used in the construction of the system. This lens was chosen for its high light transmission coefficient due to the complete filling of the air gaps between the individual lenses included in its composition.
- An optical fiber and a rod lens are precisely connected in duralumin housing. This design achieves the optimum in laser diode imaging and fiber optic compilation, ensuring low levels of signal intensity loss.
- The sample fluoresces after being irradiated by the LED. The emission signal is received at 45° from the rod lens and the emission signal is generated. It is then transmitted through the optical fiber to the detector.

3. Results

3.1. Amylolytic Activity of the Isolated Bacillus Strains

The *Bacillus* strains were used as a result of the hydrolysis tests, and they showed the ability to digest wheat starch. When using solid nutrient media, an indicator of the presence of amylolytic activity is areas of lightning around the colonies assimilating starch, which can be observed after treatment with Lugol's solution (Table 2). The most active bacteria with amylolytic activity were *B. circulans,* with a reported starch digestion area of 28.663 ± 3.163 mm, followed by *B. subtilis* with an area of 21.656 ± 2.511 mm and *B. licheniformis* 22.166 ± 2.020 mm. The lowest amylolytic activity showed *B. megaterium*—12.833 ± 1.217 mm.

Table 2. Amylolytic activity of the four *Bacillus* strains. Results are shown as means with standard deviation (n = 3).

№	*Bacillus* Strain	Amylolytic Activity, mm	
		Cultured Cells	Cell-Free Supernatant
1	*Bacillus subtilis*	21.656 ± 2.511 a	9.6 ± 0.536 a
2	*Bacillus licheniformis*	22.166 ± 2.020 a	9.7 ± 0.646 a
3	*Bacillus circulans*	28.663 ± 3.163 b*	11.2 ± 0.753 b*
4	*Bacillus megaterium*	12.833 ± 1.217 c*	8.50 ± 0.986 c*

Note: a, b, and c indicate statistical references and * indicates high significance.

3.2. Proteolytic Activity of Tested Bacillus spp. Strains

Three days after inoculation (on 72nd hour), the statistically proven most active bacteria with proteolytic activity were *B. circulans*, with a reported protein digestion area of 48.2 ± 0.803 mm, followed by *B. licheniformis* 47.6 ± 0.324 mm and *B. subtilis* 38.6 ± 0.561 mm. The lowest proteolytic activity was *B. megaterium*—28.3 ± 0.193 mm (Table 3).

Table 3. Proteolytic activity of the four *Bacillus strains*. Results are shown as means with standard deviation (n = 3).

№	*Bacillus* Strain	Proteolytic Activity. Mm					
		Cultured Cells			Cell-Free Supernatant		
		24 h	48 h	72 h	24 h	48 h	72 h
1	*B. subtilis*	24.0 ± 0.206 a	33.3 ± 0.162 a	38.6 ± 0.561 a	0	13.0 ± 0.224 b	11.8 ± 0.321 a
2	*B. licheniformis*	25.5 ± 0.218 a	38.9 ± 0.176 a	47.6 ± 0.324 b	0	6.08 ± 0.186 a	12.6 ± 0.052 a
3	*B. circulans*	32.2 ± 0.702 b*	42.4 ± 0.275 b*	48.2 ± 0.803 b*	8.0 ± 0.15	12.5 ± 0.173 b*	16.3 ± 0.820 b*
4	*B. megaterium*	23.4 ± 0.369 a	26.6 ± 0.294 a	28.3 ± 0.193 a	0	7.8 ± 0.204 a	10.3 ± 0.068 a

Note: a and b indicate statistical references and * $p < 0.01$ indicates high significance.

The higher proteolytic activity was also observed in the cell-free supernatant of *B. circulans*, with a reported protein digestion area of 16.3 ± 0.820 mm, followed by *B. licheniformis* 12.6 ± 0.052 mm, *B. subtilis* 11.8 ± 0.321 mm, and the lowest activity *B. megaterium*—10.3 ± 0.068 mm (Table 3).

3.3. Quantification of Indole-3-Acetic Acid (IAA)

The results obtained are shown in Figure 2. The four bacterial strains are weak IAA producers. From the results, it can be concluded that the bacteria have a low level of IAA synthesis of 0.585 ppm (µd/mL) in *B. subtilis*, 0.519 ppm in *B. licheniformis*, and 0.512 ppm in *B. circulans* and the significantly ($p < 0.05$) highest IAA synthesis is seen in *B. megaterium* at 0.593 ppm.

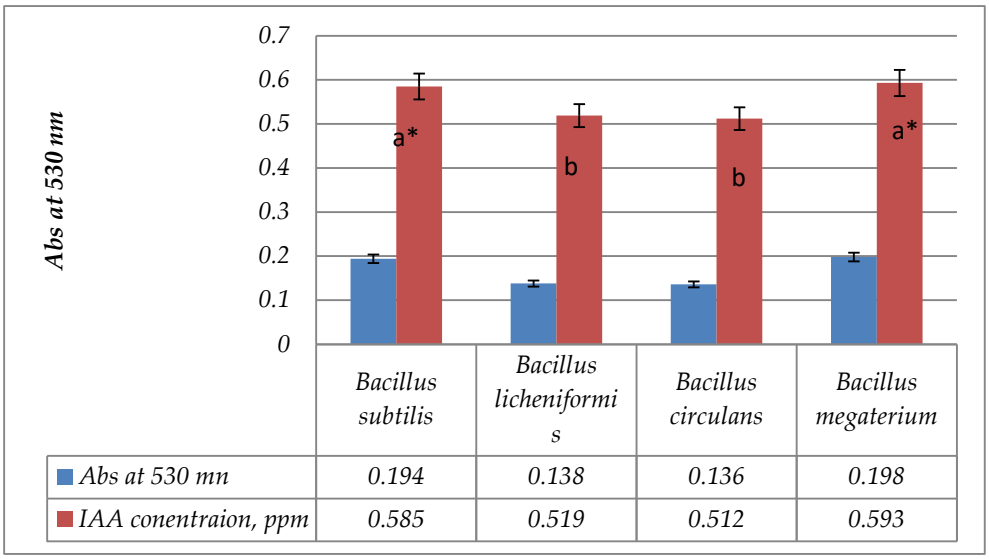

Figure 2. Indole-3-acetic acid production by the test bacteria in a medium with 0.1% (*w*/*v*) L-tryptophan incubated at 30 °C and 150 rpm for 24 h. Note: a and b indicate statistical references and * indicates high significance ($p < 0.05$).

3.4. Screening for Dissolution of Inorganic Phosphate and Zinc and Production of Siderophores

The established ability of three of the investigated strains to improve the solubility of inorganic phosphates is an essential characteristic of PGP microorganisms. The phosphate-

solubilizing index (PSI) of the studied strains was determined by a qualitative method of analysis using Pikovskaya agar (PVK). The strains were found to possess high phosphate-solubilizing activity (a solubilization zone more significant than 2 cm), measured as the phosphate-solubilization index (PSI) of the strains cultured on PVK medium. The results of the present study showed that the cell-free supernatant of *B. subtilis* and *B. licheniformis* exhibited moderate phosphate dissolution activity of 12.72 ± 159 mm and 15.23 ± 0.503, respectively (Table 4). The highest dissolution index was observed in *B. megaterium* cell-free culture (21.63 ± 0.129 mm), followed by *B. circulans* (19.60 ± 0.078 mm) (Table 4). The established ability of the investigated strains to improve the solubility of inorganic phosphates is an essential characteristic of PGP microorganisms.

Table 4. The biochemical characterization of tested *Bacillus* strains.

Bacterial Strains	Phosphate-Solubilization Index (PSI) as a Zone in mm ± SD, n = 3	Zinc-Solubilization as a Zone in mm ± SD, n = 3	Production of Siderophores as a Yellow Zone in mm on CAS Agar, n = 3
Bacillus subtilis	12.72 ± 159 a	11.50 ± 0.873 a	18.3 ± 0.07 a
Bacillus licheniformis	15.23 ± 0.503 a	9.75 ± 0.456 a	32.0 ± 0.218 b*
Bacillus circulans	19.6 ± 0.078 b	18.52 ± 1.720 b	19.0 ± 0.113 a
Bacillus megaterium	21.63 ± 0.129 b*	27.57 ± 1.792 c*	39.6 ± 0.144 b*

Note: a, b, and c indicate statistical references and * indicates high significance.

B. megaterium showed highest Zn solubilizing activity of 27.57 ± 1.792 zone of dissolution compared to the other three *Bacillus* strains as follows: *B. subtilis*—11.50 ± 0.873 mm, *B. licheniformis*—9.75 ± 0.456 mm, and *B. circulans* with a zone of 18.52 ± 1.720 mm (Table 4).

A change from blue to yellow was found in *B. subtilis* with a zone of 18.3 ± 0.075 mm, in *B. circulans* with a zone of 19.0 ± 0.113 mm, *B. licheniformis* at 32.0 ± 0.218 mm, and *B. megaterum* at 39.6 ± 0.144 mm (Table 4). Several studies revealed that siderophores are involved in the stimulation of plant growth and also in protecting the plants against various biotic stress phytopathogens [43]. In 2011, Yu et al. reported that *B. sublilis* CAS15 produced the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin, protecting peppers from *Fusarium* wilt and also enhancing the yield [44]. The siderophore-producing carrier strain *Bacillus siamensis* Gxun-6 had strong *Fusarium* wilt control and growth-promoting effects on bananas [45].

3.5. Screening Bacillus spp. for Genes Encoding Lipopeptides

In an attempt to determine whether the selected four *Bacillus* strains carry genes for the production of known lipopeptides, primers specific for individual genes were used using PCR analysis (Figure 3). In the current study, PCR analysis showed the presence of *ituC* and *fenD* in *B. subtilis*, *B. licheniformis*, and *B. megaterium* (Figure 3A,B). *ItuC* and *fenD* genes were not detected in *B. circulans*. The *srfAA* gene was detected in all *Bacillus* strains (Figure 3C). Iturins are cyclopeptides and fengycins are macrolactones characterized by L- and D-amino acids and variable hydrophobic tails. A recent investigation found that *Bacillus velezensis* LM2303 had vigorous antagonist activity against *F. graminearum* and significantly reduced the disease severity in field conditions by producing secondary metabolites with biocontrol functions like surfactin A, the siderophore bacillibactin, molybdenum cofactor, and teichuronic acid [46,47].

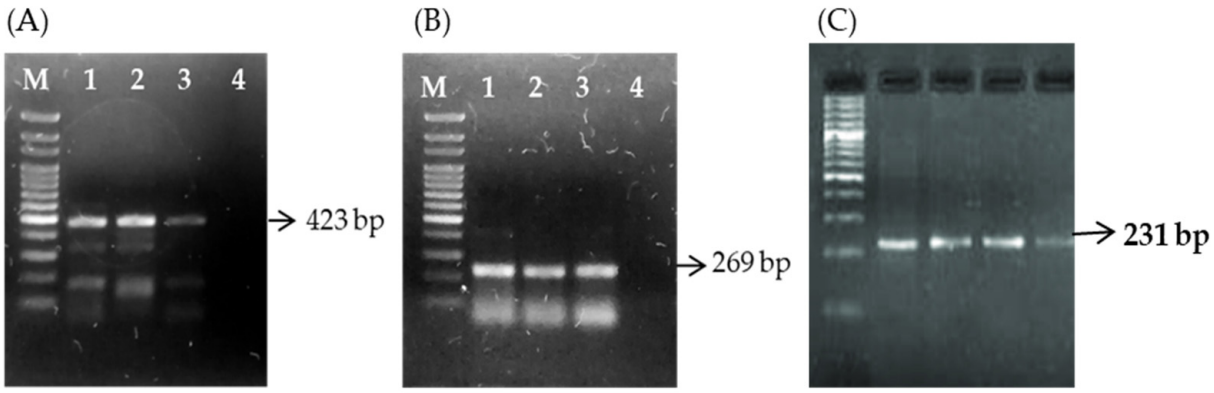

Figure 3. 1.5% agarose gel showing the PCR products from (**A**) *ituC* (423 bp), (**B**) *fen*D (269 bp), and (**C**) *surfAA* (231 bp). The marker (M) lane contains the 100 bp DNA ladder New England Biolabs (Cambridge, UK. Lane 1—*B. subtilis*, lane 2—*B. licheniformis*, lane 3—*B. megaterium* and lane 4—*B. circulans*.

3.6. Antifungal Activity of the Tested Strains

A large group of active bacterial strains have been described as biocontrol agents due to their ability to produce various antifungal metabolites [47]. The antifungal activity of the four tested strains inhibited *Fusarium graminearum* NBIMCC 2294 and the calculated inhibition effects are presented in Figure 4. *B. subtilis* exhibited a strong inhibition of mycelia radial growth of 54.20%. *B. licheniformis* and *B. megaterium* showed inhibition of 50.57% and 47.53%, respectively. According to the severity scale, those bacteria are moderate to strong inhibitors of fungal growth. A weaker inhibition rate of 33.37% was detected in the dual cultivation of *B. circulans* and *F. graminearum* 2294.

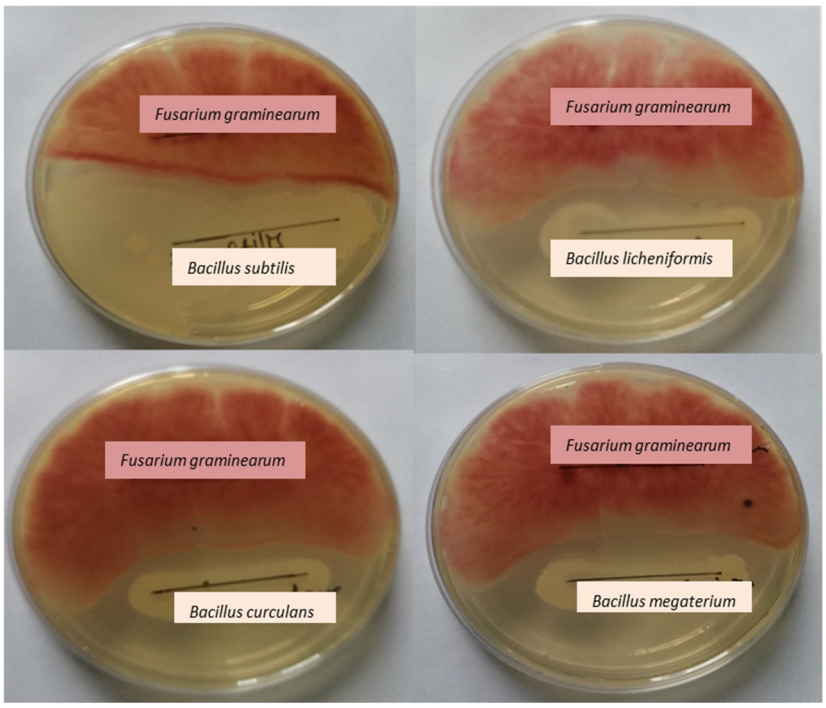

Figure 4. Antifungal activity of the tested strains against *Fusrium graminearum* NBIMCC 2294 on PDA for 7 days incubation at 28 °C.

From the obtained results, all four strains of bacilli inhibit the growth of *F. graminearum* 2294 (Figure 4). The results demonstrate the potential of *Bacillus* spp. as a biocontrol agent against *Fusarium graminearum*. The antifungal activity could be attributed to the production of secondary metabolites (e.g., lipopeptides like surfactin and iturin), competition for

nutrients and space, and/or siderophore production that limits iron availability to the fungus. Tested *Bacillus* strains are weak producers of lipopeptides and synthesize siderophores.

3.7. Influence of Tested Four Bacillus spp. on the Average Yield in t/ha of Wheat

Climate conditions during the vegetation of the common winter wheat were relatively good. The dry autumn did not allow for ordinary and timely emergence; therefore, the vegetation started after the rainfall at the end of November. The cold and dry conditions in the spring months also delayed the heading and the late precipitations at full maturity caused the final life cycle to lengthen. Severe infestations of insects at GS 11–14 and a virus transmission caused an initial thinning of the crop. The drought and the cold weather in the spring were other limiting factors for optimal development. In these conditions, the effects of the microorganisms—solely or in combination—are significant. The experiment obtained production results from the gene expression and all physiological processes and biochemical reactions to stress during crop vegetation. The grain yield was calculated after initial cleaning and threshing and is presented as t/ha. A significant increase in production was obtained in all variants. The best results were achieved when wheat was treated with *Bacillus megaterium* (42% gain) and all its combinations—*Bacillus subtilis* + *Bacillus megaterium* (33%), *Bacillus circulans* + *Bacillus megaterium* (30%), *Bacillus subtilis* + *Bacillus circulans* + *Bacillus megaterium* (28%), and *Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium* and *Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus megaterium* (26%) (Table 5).

Table 5. Influence of the four tested *Bacillus* spp. on the average yield in t/ha of wheat and increase in yield compared to the national standard in percent (%).

Field Plot Number	Variant	Average Grain Yield, t/ha	% St
1	Control standard	5.18	100
12, 29, 33	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans*	5.81	112
3, 27, 31	*Bacillus licheniformis*	5.94	115
2, 20, 40	*Bacillus subtilis* + *Bacillus circulans*	6.05	117
7, 21, 41	*Bacillus subtilis* + *Bacillus circulans*	6.08	117
16, 26, 46	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*	6.14	119
4, 18, 36	*Bacillus circulans*	6.16	119
6, 17, 35	*Bacillus subtilis* + *Bacillus licheniformis*	6.22	120
9, 19, 38	*Bacillus licheniformis* + *Bacillus circulans*	6.30	122
10, 22, 41	*Bacillus licheniformis* + *Bacillus megaterium*	6.33	122
13, 26, 39	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus megaterium*	6.53	126
15, 28, 49	*Bacillus licheniformis* + *Bacillus circulans* + *Bacillus megaterium*	6.53	126
14, 24, 37	*Bacillus subtilis* + *Bacillus circulans* + *Bacillus megaterium*	6.63	128
11, 23, 34	*Bacillus circulans* + *Bacillus megaterium*	6.73	130
8, 30, 45	*Bacillus subtilis* + *Bacillus megaterium*	6.88	133
5, 32, 43	*Bacillus megaterium*	7.36	142

3.8. Mobile Fluorescence Spectroscopy for Evaluation of the Effect of Bacillus spp. on Wheat

In Figure 5a, differences in emission wavelength for control (1), *B. subtilis* (parcel number in triplicate 2, 20, and 40), *B. licheniformis* (3, 27, and 31), *B. circulans* (4, 36, and 18), and *B. megaterium* (4, 36, and 18) are shown in three replications. The highest intensity is *B. licheniformis*, and the lowest is *B. circulans*. This fact is a prerequisite for the highest

content in the sample being *B. licheniformis* and the lowest content being in the case of *B. circulans*. Control has an average level of intensity; this is a prerequisite for insufficient or no content of bacilli.

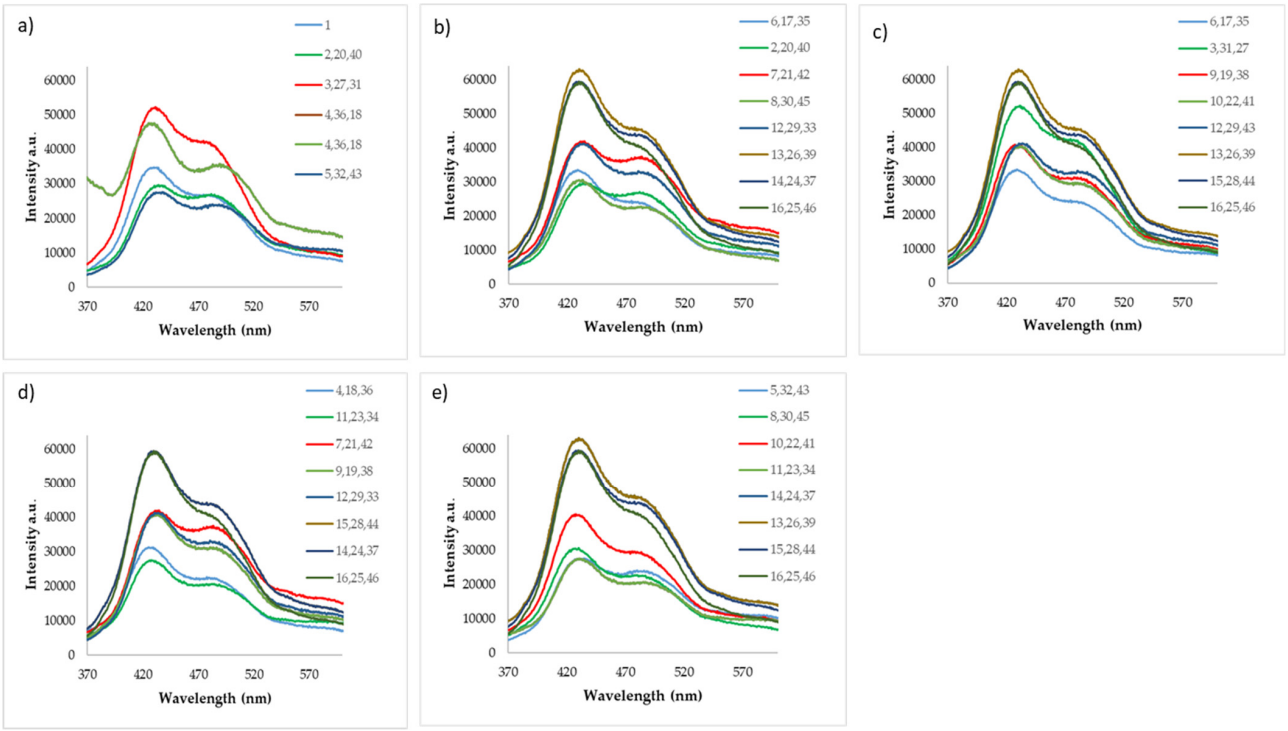

Figure 5. (**a**) Differences in emission wavelength for Control (1), *B. subtilis* (2, 20, and 40), *B. licheniformis* (3, 27, and 31), *B. circulans* (4, 36, and 18), and *B. megaterium* (4, 36, and 18). (**b**) Differences in emission wavelength for *B. subtilis* (2, 20, and 40), *B. subtilis* + *B. licheniformis* (6, 17, and 35), *B. subtilis* + *B. circulans* (7, 21, and 42), *B. subtilis* + *B. megaterium* (8, 30, and 45), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). (**c**) Differences in emission wavelength for *B. licheniformis* (3, 27, and 31), *B. subtilis* + *B. licheniformis* (6, 17, and 35), *B. licheniformis* + *B. circulans* (9, 19, and 38), *B. licheniformis* + *B. megaterium* (10, 22, and 41), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). (**d**) Differences in emission wavelength for *B. circulans* (4, 18, and 36), *B. subtilis* + *B. circulans* (7, 21, and 42), *B. licheniformis* + *B. circulans* (9, 19, and 38), *B. circulans* + *B. megaterium* (11, 23, and 34), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). (**e**) Differences in emission wavelength for *B. megaterium* (5), *B. subtilis* + *B. megaterium* (8, 30, and 45), *B. licheniformis* + *B. megaterium* (10, 22, and 41), *B. circulans* + *B. megaterium* (11, 23, and 34), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46).

Figure 5b represents the difference in emission wavelength for *B. subtilis* (2, 20, and 40) *B. subtilis* + *B. licheniformis* (6, 17, and 35), *B. subtilis* + *B. circulans* (7, 21, and 42), *B. subtilis* + *B. megaterium* (8, 30, and 45), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). The highest intensity was found in *B. subtilis* + *B. circulans* + *B. megaterium*, while the lowest was in the case of *B. subtilis*. This fact is a prerequisite for the highest

content in the sample to be the triple combination of *B. subtilis* + *B. circulans* + *B. megaterium* and the lowest content to be when *B. subtilis* + *B. circulans* have been used. The double combination of *B. subtilis* + *B. circulans* showed an average level of intensity, a prerequisite for the insufficient presence of *B. subtilis* + *B. circulans* + *B. megaterium* and the dominance of a larger amount of *B. circulans* content in the sample.

Figure 5c demonstrates the differences in emission wavelength for *B. licheniformis* (3, 27, and 31) applied alone or combined with other strains: *B. subtilis* + *B. licheniformis* (6, 17, and 35), *B. licheniformis* + *B. circulans* (9, 19, and 38), *B. licheniformis* + *B. megaterium* (10, 22, and 41), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). The highest intensity is *B. licheniformis* + *B. circulans* + *B. megaterium* and the lowest is *B. subtilis* + *B. licheniformis* + *B. circulans*. This fact is a prerequisite for the highest content in the sample to be *B. licheniformis* + *B. circulans* + *B. megaterium*, and the lowest content showed the combination of *B. subtilis* + *B. licheniformis* + *B. circulans*. *B. licheniformis* + *B. circulans* have an average level of intensity. This is a prerequisite for the insufficient presence of *B. circulans* and the dominance of a larger amount of *B. licheniformis* content in the sample.

Figure 5d shows the difference in emission wavelength for *B. circulans* (4, 18, and 36) compared to the other bacteria in a single, double, triple, and quadruple application: *B. subtilis* + *B. circulans* (7, 21, and 42), *B. licheniformis* + *B. circulans* (9, 19, and 38), *B. circulans* + *B. megaterium* (11, 23, and 34), *B. subtilis* + *B. licheniformis* + *B. circulans* (12, 29, and 33), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). The highest intensity is *B. subtilis* + *B. licheniformis* + *B. circulans* and the lowest is *B. circulans* + *B. megaterium*. This fact is a prerequisite for the highest content in the sample to be *B. subtilis* + *B. licheniformis* + *B. circulans*, and the lowest content was measured with the mixture of *B. circulans* + *B. megaterium*. *B. licheniformis* + *B. circulans* with an average level of intensity. This is a prerequisite for the insufficient presence of *B. licheniformis* and the dominance of a larger amount of *B. circulans* content in the sample

Figure 5e demonstrates the difference in emission wavelength for *B. megaterium* compared with the other three strains in different combinations (5): *B. subtilis* + *B. megaterium* (8, 30, and 45), *B. licheniformis* + *B. megaterium* (10, 22, and 41), *B. circulans* + *B. megaterium* (11, 23, and 34), *B. subtilis* + *B. circulans* + *B. megaterium* (13, 26, and 39), *B. licheniformis* + *B. circulans* + *B. megaterium* (15, 28, and 44), *B. subtilis* + *B. circulans* + *B. megaterium* (14, 24, and 37), *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46); *B. megaterium* (14, 24, and 37), and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium* (16, 25, and 46). The highest intensity is *B. subtilis* + *B. circulans* + *B. megaterium* and the lowest is *B. circulans* + *B. megaterium*. This fact is a prerequisite for the highest content in the sample to be *B. subtilis* + *B. circulans* + *B. megaterium*, and with the lowest content *B. circulans* + *B. megaterium*. *B. licheniformis* + *B. megaterium* with an average level of intensity is a prerequisite for the insufficient presence of *B. megaterium* and the dominance of a larger amount of *B. licheniformis* content in the sample.

The spectral distribution of the samples with the bacteria applied alone and in combination confirms their plant growth-stimulating activity. The intensity level of the emission signal was directly proportional to the amylolytic activity of the four Bacillus strains. The samples with the lowest signal intensity are those with *B. megaterium* and the highest are those with *B. circulans* (Figure 5). The most active samples with amylolytic activity were *B. circulans* intermediate with *B. licheniformis* and *B. subtilis*. The lowest amylolytic activity was *B. megaterium*. A higher amylolytic activity was confirmed, also observed in the cell-free supernatant of *B. circulans* followed by *B. licheniformis* and *B. subtilis*.

4. Discussion

Bacillus spp. is widely distributed in nature and associated with other organisms and normal inhabitants of soil, plants, and aquatic environments. They participate in dissolving soil-insoluble phosphates, transforming complex organic biomass into mineral compounds that plants use, and synthesizing growth factors, such as amino acids and vitamins. The presence of *Bacillus* spp. in the soil causes the enrichment of mobile phosphorus forms. Organic acids produced by these microorganisms have a positive effect on the process of dissolution of P and Zn, according to Gyaneshwar (2002) [48]. Those beneficial microbes can improve plant growth by enhancing the availability of nutrients, regulating phytohormones, and increasing plant tolerance against stresses. These results correlate with several recent investigations [49,50] for improving plant growth in maize and wheat using broth, coal, and alginate bead formulations of *Bacillus* spp. Emami described the highest grain yield in 2019 after applying three commercial microbial fertilizers (*Paenibacillus azotofixans*, *Bacillus megaterium,* and *Bacillus subtilis*) [51]. *Bacillus subtilis* is well known to produce peptide antibiotics and siderophores (bacillobactin) capable of binding iron ions by binding all available forms of iron into chelates and sharing them with plants [52]. *Bacillus megaterium* was reported to produce acids or enzymes as metabolites, making it capable of dissolving phosphorus (Saeid, 2018 [53]). *B. circulans* Xue-113168 was reported to have a high potassium dissolving rate, chitinase-producing enzymes with disease resistance effects, and an ability to increase crop yields [54]. The *Bacillus licheniformis* HSW-16 was tested for its plant growth-promoting effect on wheat (variety C-309) plants [55].

The first criterion for selecting isolated bacteria is their ability to utilize starch as the primary carbon source. The amylolytic activity on solid culture media is mainly due to cell-bound amylases. The expression of this depends on multiple factors [56]. After Lugol's staining, the maximum halo zone developed on the starch agar plates was observed with *Bacillus licheniformis* and *Bacillus circulans* (>20 mm) (Table 2).

The nature and extent of the proteolytic processes are essential for preserving good antimicrobial characteristics. The highest proteolytic activity characterizes the isolates. *Bacillus* spp. is involved in specific degradation processes of proteins that can fulfill a particular function (e.g., cell cycle), differentiation process, or stress response [57]. From the results of Table 3, all the tested *Bacillus* strains possessed the ability to hydrolyze milk casein. The potential for utilization of bacilli that produce biologically important proteases was discussed and it was suggested that they be utilized as biological control agents and to decrease the application of chemical pesticides [58].

The synthesis of microbial phytohormones by bacteria is associated with signaling changes in the root and plant growth stimulation. A phytohormone in the microbial culture supernatant cannot prove the molecule's functional role in its interaction with the plant. Several studies have observed a correlation between plant growth and hormone concentration measured in the culture medium or colonized plant tissues in experiments and in situ [35].

Phosphate solubilization is a crucial characteristic that provides bioavailable phosphate to improve plant growth [59]. The presence of phosphate-solubilizing bacterium in soils may be a positive indicator for utilizing the microbes as biofertilizers for crop production and being beneficial for sustainable agriculture [60]. Acid phosphatases and phytases synthesized by rhizosphere microorganisms are involved in the organic solubilization of soil phosphorus [59]. All the studied bacteria could dissolve phosphate compounds in soil and provide necessary metabolites during the growth phase of the wheat (Table 4).

ZnO is insoluble in water and soluble in most acids [61]. Carboxylic groups of organic acids can complex metal cations and displace anions to convert insoluble forms of Zn, such as ZnO, into more soluble forms [62]. Several laboratory studies have reported

that microbes that produce siderophores, or CO_2, may contribute to environmental Zn solubilization, depending on the species of organism and their growth conditions [63,64].

Iron is the fourth most abundant element. Bacteria require iron for several metabolic and signaling functions, including electron transport, peroxide reduction, amino acid and nucleoside synthesis, DNA synthesis, and photosynthesis. *Bacillus* spp. has developed a mechanism for iron composition by siderophore synthesis. Siderophores are small iron-chelating molecules with a peptide backbone and various coordinating iron-ligating groups. *Bacillus* spp. produces various siderophores, which play a crucial role in their existence [42,43]. In the experiment of Nithyapriya et al., 2021 [65], siderophore-producing *B. subtilis* LSBS2 significantly enhanced plant biomass, pigment content, iron, and oil content in bioinoculant-treated sesamum plants. It exhibited multiple plant growth-promoting (PGP) traits, such as hydrogen cyanide (HCN), ammonia, and indole acetic acid (IAA), and solubilized phosphate *Bacillus* spp. is known to stimulate plant growth and prevent pathogen infection [65] and also plays a significant role against biotic stresses through the production of phytohormones, volatile organic compounds, exopolysaccharides, siderophores, and P solubilization [66]. Climate conditions during the vegetation of the winter wheat were relatively good. The dry autumn did not allow for regular and timely emergence; therefore, the vegetation started after the rainfall at the end of November. The cold and dry conditions in the spring months also delayed the heading and the late precipitations at full maturity caused the final life cycle to lengthen. Severe infestations of insects at GS 11–14 and a virus transmission caused an initial thinning of the crop. The drought and the cold weather in the spring were other limiting factors for optimal development. In these conditions, the effects of the microorganisms—solely or in combination—are significant. The experiment obtained production results from the gene expression and all physiological processes and biochemical reactions to stress during crop vegetation.

F. graminearum is one of the pathogens that can infect wheat florets in the soft dough stage of kernel development [67]. It also produces mycotoxins, which are unsuitable for human consumption. Fusarium head blight disease has become one of the most economically important and studied plant pathogens. The environmental pollution caused by excessive use of chemical pesticides has led to considerable changes in people's attitudes toward their substation with biological control with antagonistic microorganisms [68].

Iturins, fengycins, and surfactins are nonribosomal biosynthesized peptides [69]. Iturins are cyclopeptide antibiotics, while fengycins and surfactants are macrolactones characterized by L and D amino acids and variable hydrophobic tails. In the current investigation, the four *Bacillus* strains displayed solid antifungal activity against *F. graminearum* in vitro (Figure 5). These results were similar to those reported by Gong, 2015 [69], that iturins strongly inhibited *F. graminearum.* Climate change and observed dry and hot summers in recent years are the reasons for limiting the spread of the Fusarium pathogen in field conditions, and only the influence of bacteria on wheat yield was reported.

The dual culture assay confirmed the antagonistic potential of *Bacillus* spp. against *F. graminearum*. Future studies should focus on identifying the exact antifungal compounds and conducting field trials to validate these findings under agricultural conditions. Variations in inhibition percentages between different Bacillus strains highlight the importance of strain selection for biocontrol applications.

Microbial bioproducts also have other positive effects on plants by promoting plant growth and acting by directly improving plant nutrition, either by solubilizing nutrients (phosphates and zinc) or by fixing atmospheric nitrogen [70]. They play a crucial role in interacting with the host plant and other beneficial organisms [71]. *Bacillus species* are well-studied organisms and the "generally regarded as safe" (GRAS) status has been given to *Bacillus subtilis,* which is thus recognized as non-pathogenic [72]. The commercial use

of this bacterium in agriculture is limited due to difficulties in preparing stable and long-living biological agents, according to [9]. The yield was significantly higher, as shown by a single combination of strains *B. megaterium*, followed by a triple combination of *B. subtilis*, *B. circulans*, and *B. megaterium* (Supplementary Figure S2). It was more effective in uninoculated control under pot and field experiments. This might be due to colonizing the hair and cortical cells, enhanced root surface area, and consequently, more acquisition of nutrients and plant hormones [72]. Akhtar et al. (2013) also reported a similar finding due to efficient bacterial strains being inoculated with wheat grains that enhanced the plant growth-promoting activity [73]. The application of PGPR significantly increased plant height by 25% over an uninoculated control, as reported by Abbasi et al. (2011) [74]. Inoculation with *B. megaterium* enhanced wheat yield by 42% compared to the national standard in percent (Table 5). The double combination of *B. subtilis* and *B. megaterium* resulted in a yield increase of 33%. In contrast to previously published data by [75], triple and quadruple combinations of the co-inoculated bacilli strains gave similar but lower results in Teres wheat.

Similarly, Turan et al. (2012) reported that a single inoculation with *B. megaterium* M3 increased grain yield by 19%, while a mixed inoculation with *Bacillus subtilis* OSU-142, *B. megaterium* M3, or *Azospirillum brasilense* Sp245 increased grain yield by 33% relative to noninoculated plants [75]. Food can contain various toxins produced as contaminants by pathogenic fungi (mycotoxins). Toxins can also be transferred into food through antibiotics (penicillin) or additives (aspartame). Cereals and their processed products can be tested for toxins by fluorescence spectroscopy [76]. The spectral distribution of the emission signal in wheat mainly consists of two maxima in the visible range. The intensity and shape of the fluorescence emission spectrum at room temperature depend primarily on the concentration of the fluorophore compounds and, to a lesser extent, on the structure, photosynthetic activity, and the characteristics and arrangement of the cells in the tissue [77,78]. Fluorescence spectroscopy in the food industry is widely used for quantitative analysis. It is sensitive and specific enough to detect even tiny concentrations of compounds [79,80]. For example, changes in the structures of proteins, carbohydrates, and lipids in oils can be detected through it. This is useful for verifying the authenticity of food products [81,82]. Advances in fiber optic technology offer outstanding opportunities for developing a wide range of susceptible fiber optic sensors in many new application areas. Fiber-optic components are successfully adapted to assemblies with microoptic elements such as lenses, mirrors, prisms, and gratings [83,84]. Fluorescence spectroscopy in agricultural sciences is applied to analyze tomatoes [85] and cereals [86]. Their characterization through this technique is performed by grouping objects with similar characteristics to establish methods related to their classification.

5. Conclusions

This article showed positive results with single and co-inoculations of four *Bacillus* spp. (*B. subtilis*, *B. licheniformis*, *B. circulans*, and *B. megaterium*) on the growth and development of common wheat. Despite adverse climatic conditions during winter wheat vegetation (e.g., drought, cold spring, and late precipitation), the *Bacillus* spp. improved crop resilience and productivity. This reinforces the importance of additional research to elucidate the interactions between microorganisms, foreseeing the production of biological fertilizers and biological control agents. The two most abundant lipopeptides (iturin and fengycin) produced by the tested bacteria *B. subtilis*, *B. megarerium*, and *B. licheniformis* strains showed antagonistic mechanisms against phytopathogenic *F. graminearum*. Similar to previous studies [87,88], fluorescence spectroscopy has been effectively used to examine and identify relationships or similarities between the bacterial species *Bacillus subtilis*,

Bacillus licheniformis, *Bacillus circulans*, and *Bacillus megaterium*. After the conducted experiments, it can be concluded that it is not appropriate to use combinations with *B. licheniformis* (*B. licheniformis* + *B. circulans* + *B. megaterium* and *B. subtilis* + *B. licheniformis* + *B. circulans* + *B. megaterium*) together when experiments are conducted with common wheat. The most suitable combination for treating wheat seeds is *B. megaterium*, which is applied by soil drench, as well as the combination of *B. subtilis* + *B. megaterium*, followed by a triple combination of *B. subtilis* + *B. circulans* + *B. megaterium*. The fluorescence spectroscopy results proved that the triple combination of *Bacillus subtilis*, *Bacillus circulans*, and *Bacillus megaterium*, and *Bacillus licheniformis* showed the best stimulation of development, expressed as a comparative evaluation of the yield compared to the untreated control variant. The four strains were investigated for their potential application as a biocontrol agent against *Fusarium graminearum*. The four *Bacillus* strains can also promote plant growth by affecting nutrition, root structure, and plant health and they can dissolve phosphates and zinc.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb16010001/s1, Figure S1: Regression curve equation used to calculate the IAA concentration; Figure S2: Photographs from the field experiments showing the experimental plots with different treatments of applied four Bacillus strains and measuring process. These images provide visual evidence of the experimental conditions and treatments used during the study.

Author Contributions: Conceptualization, M.P., M.M. and S.S.; methodology, M.P., M.M., A.-L.P. and V.S.; Mobile fluorescence spectroscopy software, V.S.; microbiological analysis, M.P., A.-L.P. and S.S.; filed experiments, M.M.; writing—original draft preparation, M.P., M.M., V.S. and S.S.; Funding S.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: The authors would like to express their gratitude to LB Lact Bas Laboratory for their generous support in purchasing and providing the bacterial strains Bacillus subtilis NBIMCC 2353, Bacillus megaterium NBIMCC 8947, and Bacillus circulans NBIMCC 1042, which were essential for the successful completion of this study.

Conflicts of Interest: Author Antonia-Lucia Petrova was employed by the companies Laboratory LB Lact Bas Ltd., Plovdiv, Bulgaria and BIOBACT, Yagodovo, Bulgaria. Antonia-Lucia Petrova and the remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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