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Evaluation of *Bacillus velezensis* Biocontrol Potential against Fusarium Fungi on Winter Wheat

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Abstract: Fungi of the genus *Fusarium* are economically significant pathogens in most wheat-growing regions worldwide. The biocontrol agents *Bacillus velezensis* BZR 336 g and BZR 517 were tested for growth inhibition of *F. graminearum* BZR 4. The results demonstrated that the strains are capable of deforming and destroying hyphae. The modified bioautography technique showed that the strains produce iturin A and surfactin, which probably explains the mechanism of pathogen inhibition. Furthermore, lipopeptides were detected and identified in two samples by the HPLC-HRMS. Compounds such as surfactin and their isomers and homologues were found in both samples. An experiment on an artificial infectious background in a climatic chamber established that the biological effectiveness of strains is close to that of chemical and biological references. Cultivation of plants with *B. velezensis* showed that the strains are likely to reduce the stress load. An efficacy of up to 45.0% was determined for bioagents BZR 336 g and BZR 517 in field trials, while the yield was up to 7.9 t/ha. The use of *B. velezensis* BZR 336 g and BZR 517 as biocontrol agents provides an environmentally friendly approach to the control of *Fusarium* rots on wheat, reduction of the pesticide load, and hence quality harvest.

Keywords: *Bacillus velezensis*; *Fusarium graminearum*; biocontrol; winter wheat

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Citation: Asaturova, A.M.; Zhevnova, N.A.; Tomashevich, N.S.; Sidorova, T.M.; Homyak, A.I.; Dubyaga, V.M.; Nadykta, V.D.; Zharikov, A.P.; Kostyukevich, Y.I.; Tupertsev, B.S. Evaluation of *Bacillus velezensis* Biocontrol Potential against Fusarium Fungi on Winter Wheat. *Agronomy* **2022**, *12*, 1956. https://doi.org/10.3390/agronomy12081956

Academic Editors: José David Flores-Félix and Zaki Saati-Santamaría

Received: 15 July 2022
Accepted: 10 August 2022
Published: 19 August 2022

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1. Introduction

Winter wheat (*Triticum aestivum* L.) plays a significant role in the global economy. It is one of the most important staple foods worldwide. Crop production cereals losses caused by plant diseases have reached 20% [1]. Therefore, wheat protection is a major goal for food security.

Fungi of the genus *Fusarium* are distributed in cultivated areas throughout the world. They affect roots, stems, and ears, decreasing the yield and deteriorating grain quality. This is further exacerbated by the fact that fungi of the genus *Fusarium* produce a wide range of trichothecene mycotoxins (deoxynivalenol, zearalenone, fumonisin, etc.) harmful to humans and animals [1–5].

F. graminearum is capable of causing both root rot and FHB. It has a high virulence and is dominant in warm and humid climates [4]. These are the ones that prevail in Southern Russia, where winter wheat is a major cereal crop.

The development of the disease is influenced by sowing date, variety selection, sowing density, and number of weeds [4,5]. Planting date management, with an emphasis on early planting, has been shown to be important in controlling *Fusarium* disease in wheat and maize [6,7].

Plant residues are one major source of infection with *Fusarium* in most crops. The development of infection contributes to the predominance of cereals and corn in the crop

rotation. They decompose slowly and therefore may be present in subsequent crops for at least two years [4,5]. The composition of the species that are involved in the infectious process is dynamic. Therefore, relationships between *Fusarium* species may change over time during the infection [4,8]. The dominant species may vary by crop type, geographic region, and environmental conditions [4].

Current *Fusarium* control measures include the rotation with non-host crops, the use of resistant varieties, and the use of chemical fungicides [4,6,7,9]. However, the use of chemical plant protection products causes serious damage to the environment and consequently a negative public outcry [3,10]. It is important to note that the effectiveness of chemical fungicides varies for several reasons. *Fusarium* species have different susceptibility to fungicide treatments [4,9]. Some fungicide active ingredients, such as strobilurins, lead to an increase in mycotoxins in harvested grain [5,11,12].

Microorganisms in biopreparations can provide plants with nutrients, facilitate the mobilization of macro- and microelements, improve plant health, stimulate growth and development, and control diseases, as well as improve microbiological composition and soil properties [8]. The use of beneficial microbes can improve plant defense and prevent infection with several pathogens. Plant inoculation with such microbes can confer resistance to a spectrum of pathogens or pests [1,3,10,11].

Bacillus spp., *Pseudomonas* spp., and some mycorrhizal fungi are among various microorganisms for biocontrol [13].

Kim et al. [14] showed that *Lysobacter antibioticus* HS124 inhibits the growth of *F. graminearum* BZR 4 mycelium (74.66%) in the dual culture system. Microscopic investigation showed that different concentrations of culture filtrate (10%, 30%, and 50%) from HS124 were responsible for abnormal hyphal structures, including swelling and distortion on potato dextrose agar.

Bacillus sp., isolated from wheat anthers, prevented the development of *F. graminearum* BZR 4 on wheat ears and reduced the content of deoxynivalenol in grain [15]. *B. subtilis* isolates inhibited the growth of *F. graminearum* BZR 4 by 42.8–55.0%. The development of *Fusarium* head blight (FHB) in winter wheat was reduced by 36.7% [16]. *B. subtilis* strain SG6 proved to have a high antagonistic effect against *F. graminearum* BZR 4 (72.7–87.9%). It reduced both the number of pathogenic spores (83.7–95.5%) and the level of deoxynivalenol in wheat under field conditions [17].

Bacteria of the genus *Bacillus* are effective biocontrol agents, primarily due to their production of various cyclic lipopeptides. Cyclic lipopeptides are mainly represented by the families of iturins, surfactins, and fengycins and provide plant protection through many unique mechanisms [3,13,18]. Iturin and fengycin have antifungal activity [3]. Iturins disturb cell membranes by forming pores in them and causing leakage of vital constituents [19]. Surfactins do not show noticeable fungitoxicity but are known mainly for their biosurfactant activity [20].

Cyclic lipopeptides play a key role in the induction of microbial immunity in plants. They provide protection against a wide range of pathogens and give strains an advantage in certain ecological niches [13,18,21].

In this paper, we evaluate the biocontrol potential of *B. velezensis* strains against FHB, as well as study the interaction mechanism between the strain and the pathogen.

2. Materials and Methods

2.1. Bacterial and Fungal Strains

Following comparative bioinformatic analysis, two genomes were assembled and deposited at NCBI GenBank: *B. velezensis* BZR 336 g_n (assembly: GCA_009683125.1, GenBank: NZ_WKKU00000000.1) and *B. velezensis* BZR 517_n (assembly: GCA_009683155.1, GenBank: NZ_WKKV00000000.1) [22,23].

For each experiment, fresh liquid cultures were prepared on the original optimized nutrient medium. The original optimized culture medium was derived from Czapek Medium with corn extract as nitrogen source and molasses as carbon source. The incubation

period of strain BZR 336 g was 48 h at 25 °C, and for BZR 517 it was 36 h at 30 °C [24]. Incubation was performed on a shaker (New Brunswick Scientific Excella E25, Eppendorf, Hamburg, Germany) at 180 rpm.

We used the strains of two pathogenic fungi—*Fusarium graminearum* Schwabe strain BZR 4 and *F. oxysporum* var. *orthoceras* App. et Wr. BZR 6.

Bacterial and fungal strains were obtained from the bioresource collection of the Federal Research Center of Biological Plant Protection "State Collection of Entomoacariphages and Microorganisms". In this research, we used the scientific equipment «Technological line for obtaining microbiological plant protection products of a new generation» (http://ckp-rf.ru/usu/671367, accessed on 9 August 2022).

2.2. Study of Strain–Pathogen Interaction

2.2.1. Dual Culture Method with Microscopy

The double culture method was used to identify antagonism against *F. graminearum* BZR 4. An agar block with fungal mycelium was placed on PD. The bacterial strain was applied as a strip at a distance of 6 cm from the block. The cultures were incubated for 20 days at +28.0 °C. Pure cultures of the fungal pathogen and bacteria placed separately were used as controls [25].

For microscopy, culture plates filled with an optimized nutrient medium were used with glass slides inside. The bacterial culture was placed on a glass slide on one side and the mycelium of *F. graminearum* BZR 4 on the other (Figure 1).

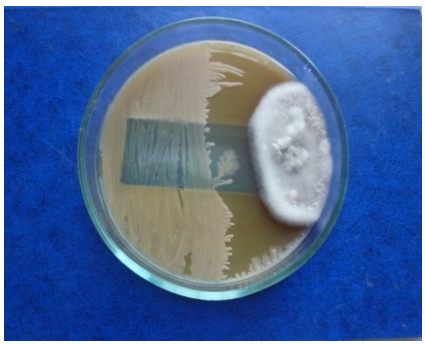


Figure 1. Method of double cultures with a glass slide for microscopy.

PDs were incubated at 24–26 °C in the light. The slides were not stained. Microscopic observations in the pathogen-antagonist system, as well as measurements of the length and diameter of mycelial segments, were carried out in AxioVisionRel (CarlZeiss AxioScopeA1, Jena, Germany) 4.8 in dynamics on the third, seventh, tenth, and fourteenth days of cocultivation.

2.2.2. Study of Composition and Quantity of Bacterial Metabolites

The composition and quantity of metabolites produced by *Bacillus velezensis* were determined visually by bioautography (Figure 2).

The technique consisted of two stages: (1) isolation and purification of bacterial exometabolites; (2) evaluation of the antifungal activity of isolated bacterial metabolites.

Isolation and purification of bacterial exometabolites.

The liquid culture of *B. velezensis* was centrifuged at 10,000 rpm for 30 min (Eppendorf, 5810R, Hamburg, Germany).

The supernatant was mixed with ethyl acetate (1:3) and stirred on a shaker for 1 h at room temperature. The ethyl acetate extract was evaporated on an RV 10D S99 rotary vacuum evaporator (IKA, Staufen im Breisgau, Germany) until a dry residue appeared. The dry residue was washed off with a specify of ethyl acetate. The isolation and analysis of the synthesized metabolites were performed using ascending thin-layer chromatography (TLC)

on Kieselgel 60 chromatographic plates (Merck, Darmstadt, Germany), layer thickness 2 mm, solvent: ethyl acetate/ethanol/water (40:15:15).

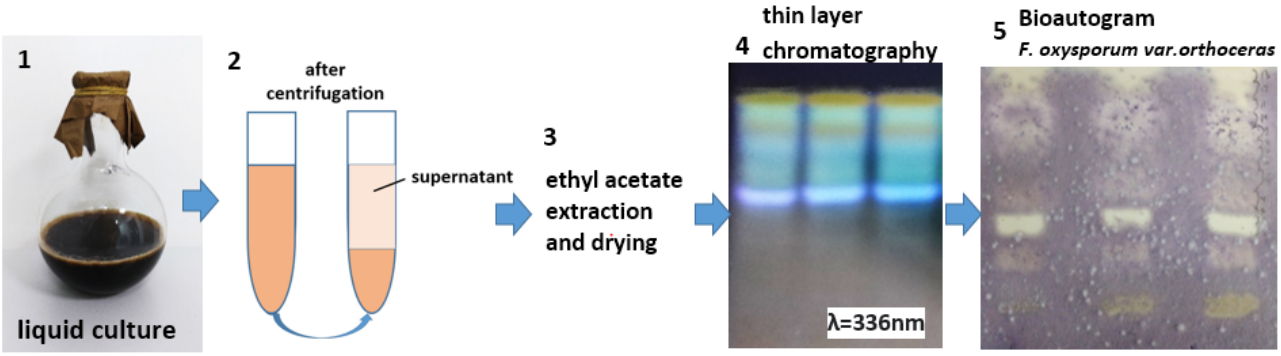


Figure 2. Main stages of the study of composition and quantity of bacterial metabolites.

During the research, commercial lipopeptides were used: surfactin, iturin A, and fengycin (Sigma-Aldrich, St. Louis, MO, USA). After TLC, the plates were analyzed at UV 366 nm (CAMAG 4, Muttenz, Switzerland) [26].

Bioautography. Bioautography was used to assess the antifungal activity of isolated bacterial metabolites. *F. oxysporum* var. *orthoceras* BZR 6 was used as a test culture. This fungus is a widespread causative agent of wheat common root rot. Moreover, this test culture is methodically acceptable, since mycelium growth pattern of this fungus provides a clear picture on bioautogram.

TLC plates were sprayed with potato-glucose nutrient medium after removing traces of solvents. After that, the test fungus suspension (*F. oxysporum* var. *orthoceras* BZR 6) was applied. Then, the plates were incubated in a humid chamber for 48 h at 28 °C. Active components were identified by the formation of zones of no/retardation growth of the fungus [27].

2.2.3. Mass Spectrometry and Sample Preparation

Sample preparation for LC-MS. In total, 1 mL of each liquid culture was centrifuged at 12,500× *g* for 20 min. The supernatant was transferred to a new tube and diluted 10 times. Further sample preparation was carried out using solid-phase extraction on C18 cartridges. The cartridges were activated with 1 mL of methanol and equilibrated with 2 mL of 2% formic acid in water followed by application of the sample. The samples were then washed with 2 mL of aqueous 2% formic acid. The final elution of samples from the cartridges was carried out with 1 mL of acetonitrile.

Reagents and materials. The mobile phase for HPLC was prepared from acetonitrile (Merck, Darmstadt, Germany), formic acid (Merck, Darmstadt, Germany), and deionized water obtained using a Milli-Q system (Millipore, Molsheim, France).

LC-MS conditions. All experiments were performed on a modified Q Exactive Orbitrap mass spectrometer (Thermo) with installed ion funnel, fore vacuum matrix-assisted laser desorption ionization (MALDI) source, and ESI source (Spectroglyph Company, Kennewick, WA, USA) coupled with Nano Spray Flex source and Dionex UltiMate 3000 (Thermo) HPLC system. We used a Waters nanoEase M/Z HSS T3 (100 × 0.075 mm, 100 Å, 1.8 µ) C18 column. Mobile phase A: 0.1% formic acid in water (H_2O); mobile phase B: 0.1% formic acid in acetonitrile (ACN) delivered as the following gradient at a flow rate of 0.60 µL/min: 0–1 min 50% B; 1–20 min linear growth 50–95% B; 20–25 min 95% B; 25–26 min linear decrease 95–50% B; 26–33 min 50% B. The column temperature was 45 °C. The injection volume was 1 µL.

Mass spectra were recorded by Orbitrap with the resolving power 70,000 (for 200 m/z) in positive ESI mode. The ESI voltage was 2.5 kV, the capillary temperature of Spectroglyph source was 150 °C, and the pressure was 6.3 Torr. Screening for metabolites was performed

by full MS followed by data-dependent analysis (DDA) in positive ionization mode. All the results from the samples were compared with those from corresponding blanks.

2.3. Climate Chamber Experiments Background

In this experiment, we used winter wheat of the variety Batko. The variety Batko is resistant to stem rust, moderately resistant to septoria and powdery mildew, moderately susceptible to brown and yellow rust, ear fusarium, and has increased frost resistance and high drought resistance (state register breeding achievements approved for use, Ministry of Agriculture of the Russian Federation, 2022). For an artificial infectious background, we used a pure culture of *F. graminearum BZR 4* grown on sterile winter wheat grain. The plants were grown in glasses with sand. The grain infected with the fungus was ground into powder and mixed with the sand, in which the seeds of winter wheat were then sown. One glass contained 300 g of sand and 5 g of inoculum. The experiment was repeated three times.

Seeds were treated with liquid cultures of strains BZR 336 g and BZR 517 before sowing. Kinto Duo, SC (triticonazole 20 g/L + prochloraz 60 g/L) was used as a chemical reference, and Fitosporin-M, L (*B. subtilis* 26 D) was used as a biological reference. The titer of the liquid culture of *B. velezensis* BZR 336 g was 3.13×10^9 CFU/mL and of *B. velezensis BZR* 517 was 4.06×10^7 CFU/mL.

Wheat plants were grown in a climate chamber KWWF 720 (Binder, Tuttlingen, Germany) at 24 °C, humidity 65%, and constant illumination at 14,200 lux for 15 days. Watering was carried out with distilled water. Root rot damage was measured by Dolzhenko scale [28]: 0—no signs of damage; 1—there are separate areas of brown color on primary and secondary roots; 2—stem base is whitish or slightly brown, individual roots or significant portions of them are brown; 3—stem base is dark with interception, most of the roots have died; 4—complete death of the stems.

2.4. Field Research

Field tests were carried out under conditions of stationary crop rotation in 2012–2015 at the FRCBPP experimental base (Krasnodar, Russia, 45.047556, 38.876061). The winter wheat variety Kalym was used after the predecessor of alfalfa. The size of the experimental plot was 10 m^2. Each experiment was performed in triplicate. The total area of the experiment was 150 m^2.

The grain was treated with liquid cultures of *B. velezensis* BZR 336 g and BZR 517 a day before sowing. Application rate of BZR 336 g was 3 l/t and of BZR 517 was 2 l/t. The flow rate of the working fluid was 10 l/t. In the control variant, the seeds were treated with tap water.

Sowing was carried out with a mechanical grain seeder (Astra SZ-3.6., USSR). Seeding rate was 220 kg/ha and row spacing was 15 cm. We preventively treated vegetative plants twice during the growing season. Application rate of BZR 336 g was 3 L/ha and of BZR 517 was 2 L/ha. The flow rate of the working fluid was 300 L/ha. Titer of liquid culture *B. velezensis* BZR 336 g was 2.1×10^9 CFU/mL and of *B. velezensis* BZR 517 was 2.5×10^9 CFU/mL. Raxil, KS (tebuconazole, 60 g/L) for seed treatment and Alto Super, KS (propiconazole + cyproconazole, 250 + 80 g/L) for plant treatment were used as a chemical reference. As a biological standard, we used Fitosporin-M, L (*B. subtilis* 26 D). In 2012–2013, no biological standard was used.

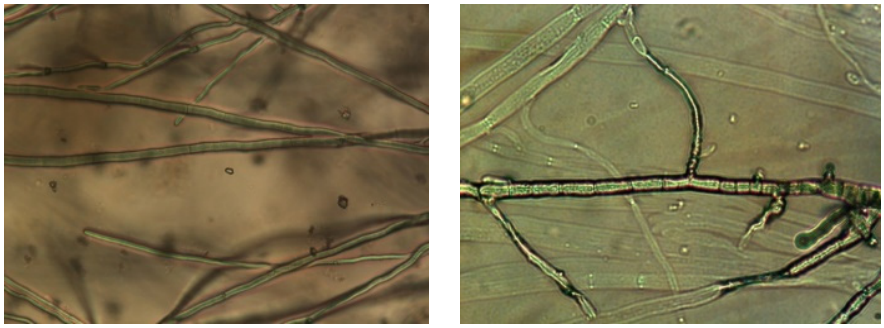
During the year, we checked the *Fusarium* root rot complex at various stages of plant development. Here, we present the results obtained at the maturity stage (Z 73–77). Biological efficiency (BE) was determined by the Abbott formula [29].

3. Results

3.1. Study of Strain–Pathogen Interaction

3.1.1. Modified Dual Culture Method

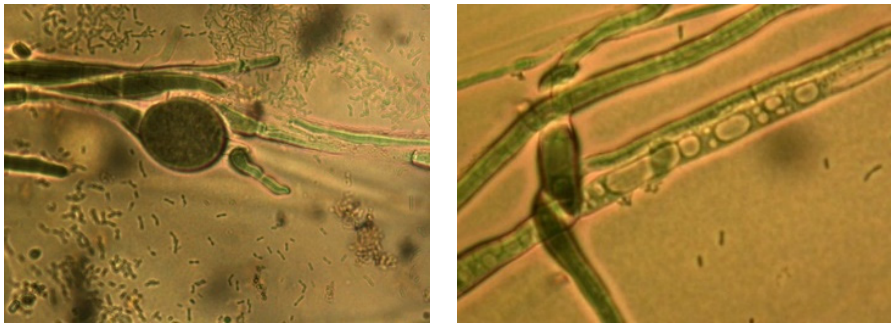
We observed shortening and thickening of mycelium segments during co-cultivation of a pure culture of *F. graminearum* BZR 4 with the studied strains *B. velezensis* (Figure 3).



(**a**) (**b**)

Figure 3. *F. graminearum* BZR 4 hyphae in control without antagonist (**a**) and in co-cultivation with *B. velezensis* BZR 336 g (**b**), ×200 magnification.

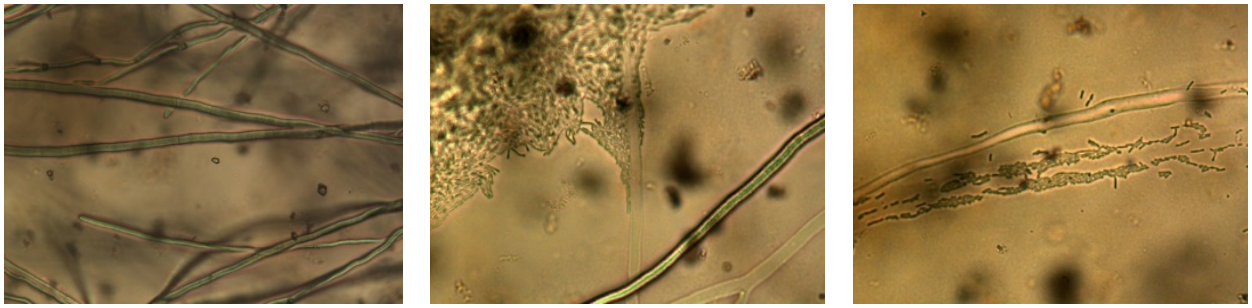
In addition, some segments of the mycelium of *F. graminearum* BZR 4 took on a spherical shape (similar to cells of the "chlamydospore" type). Often this was accompanied by cell wall integrity defects and ultimately the death of ruptured hyphal cells. Degenerative changes in intracellular membrane structures ("vacuolization") were also observed, resembling damage caused by osmotic stress (Figure 4).



(**a**) (**b**)

Figure 4. Pathological changes in *F. graminearum* BZR 4: cells of the chlamydospore type (**a**), vacuolization of the hyphae contents (**b**), magnification ×400.

On days 7–14, we registered that bacteria BZR 336 g interacted with hyphae of *F. graminearum* BZR 4, apparently using the latter as a food source. Bacterial chemotaxis towards the hyphae, hyphae encirclement by bacterial cells (Figure 5b), and gradual lysis (Figure 5c) were recorded.

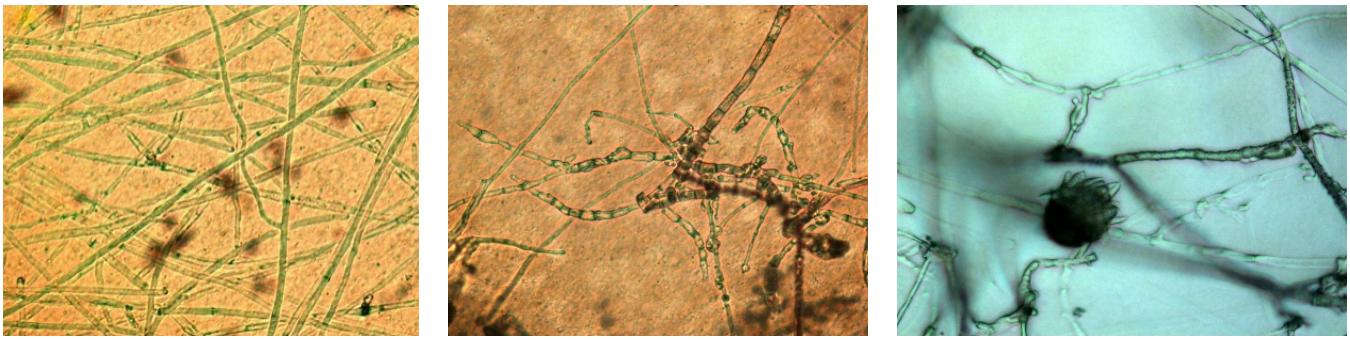


(**a**) (**b**) (**c**)

Figure 5. *F. graminearum* BZR 4: (**a**)—control (*F. graminearum* BZR 4 without antagonist bacterium); (**b**)—colonization of *B. velezenzis* BZR 336 g hyphae *F. graminearum* BZR 4; (**c**)—hyphae lysis of *F. graminearum* BZR 4 during incubation with antagonist bacterium *B. velezenzis* BZR 336 g, ×400 magnification.

When *F. graminearum* BZR 4 was co-cultivated with BZR 517, similar pathological changes in the mycelium were observed, as in cultivation with strain BZR 336 g. However, they were noticeably less pronounced. The formation of chlamydospore type cells was not observed and the "vacuolization" of hyphae was less pronounced. No active growth of BZR 517 towards the fungus culture was recorded; chemotaxis to hyphae was observed mainly during random sieving of bacterial cells.

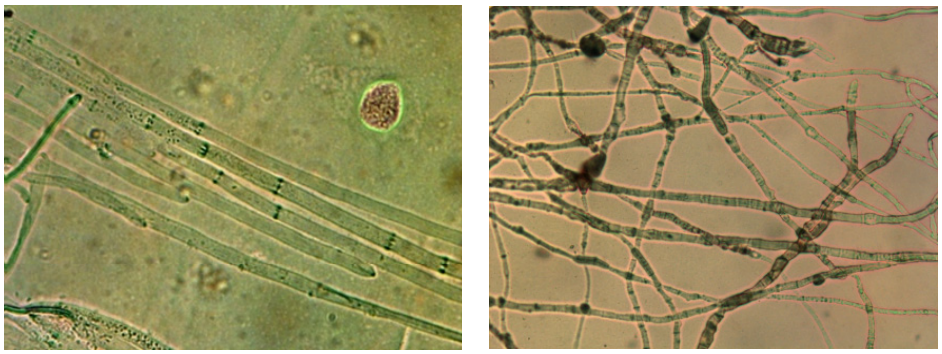
In response to the co-cultivation with bacterial strain BZR 517, we observed shortening of hyphae and an increased branching (Figure 6b). After 14 days of co-cultivation, the fungus intensively formed conidia (Figure 6c). This was not observed either in the control or in the BZR 336 g variant.



(**a**) (**b**) (**c**)

Figure 6. *F. graminearum* BZR 4 without an antagonist bacterium (**a**), shortening and hyphae branching (**b**), and conidia formation (**c**) when co-cultivated with *B. velezensis* BZR 517, ×400 magnification.

During the experiment, we recorded two fundamentally different types of hyphae damage: (1) gradual dissolution without shape loss, where the hypha remains long and even but becomes transparent and dissolves over time (Figure 7a); (2) change in hyphae shape, which was expressed as shortening, swelling, and increased branching, where the hypha remains pigmented but shortens and thickens (Figure 7b).



(**a**) (**b**)

Figure 7. Types of *F. graminearum* BZR 4 damage: (**a**)—gradual lysis of hyphae without shape loss; (**b**)—degradation of hyphae by twisting, shortening, and curvature, ×400 magnification.

3.1.2. Analysis of Bacterial Metabolites by the Modified Bioautography Technique

UV analysis of the isolated metabolites (366 nm) on a thin-layer plate showed that both strains *B. velezensis* BZR 336 g and BZR 517 produce a large number of compounds. These metabolites differ both in chromatographic mobility and in the nature of the luminescence. This probably indicates differences in chemical structures (Figure 8).

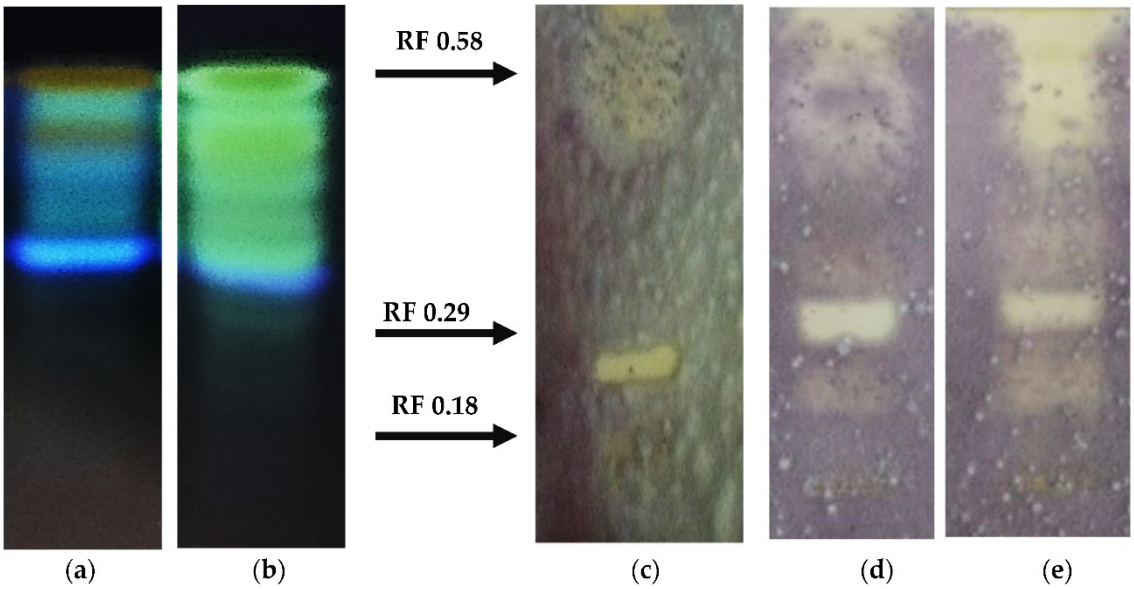


Figure 8. Thin layer chromatograms of ethyl acetate extracts of supernatants of *B. velezensis* BZR 336 g (**a**) and *B. velezensis* BZR 517 (**b**) and UV 366 nm and bioautograms of commercial lipopeptides (**c**) of ethyl acetate extracts of supernatants of *B. velezensis* BZR 336 g (**d**) and *B. velezensis* BZR 517 (**e**) with identified lipopeptide profiles: Rf 0.58—surfactin; Rf 0.29—iturin A (test culture of *F. oxysporum* var. orthoceras BZR 6).

We compared the resulting lipopeptide profiles with commercial surfactin and iturin A and found that both strains produced both the surfactin (RF 0.58) and the iturin A (RF 0.29). Fengycin (RF 0.18) inhibits the growth of the test fungus to a lesser extent. Hence, it is difficult to evaluate its antifungal activity by this method. In general, visual assessment leads to the conclusion that *B. velezensis* BZR 336 g accumulates more surfactin and iturin A than *B. velezensis* BZR 517 (Figure 8).

3.1.3. Identification of the Structure of Bacterial Metabolites by Mass Spectrometry

The use of HPLC-HRMS made it possible to detect and identify lipopeptides in samples 316 g and 517 (Table 1).

Table 1. Observed (m/z_{ob}) and theoretical (m/z_{th}) masses of mass signals obtained for surfactin, iturin, and their homologues by HPLC-ESI+ HRMS.

Retention Time, RT	Sample No.	m/z_{ob}	m/z_{th}	Ionization Mode	Molecular Formula	Error (ppm)	Assignment
19.07	BZR 336 g	994.6301	994.6435	$[M + H]^+$	$C_{50}H_{87}N_7O_{13}$	13.5	Surfactin A-CH_2
20.09	BZR 336 g	1008.6451	1008.6591	$[M + H]^+$	$C_{51}H_{89}N_7O_{13}$	13.9	Surfactin A
21.75	BZR 336 g	1022.6611	1022.6748	$[M + H]^+$	$C_{52}H_{91}N_7O_{13}$	13.4	Surfactin B
22.69	BZR 336 g	1036.6768	1036.6904	$[M + H]^+$	$C_{53}H_{92}N_7O_{13}$	13.1	Surfactin C
23.84	BZR 336 g	1050.6950	1050.7061	$[M + H]^+$	$C_{54}H_{94}N_7O_{13}$	10.6	Surfactin C + CH_2
19.18	BZR 517	994.6307	994.6435	$[M + H]^+$	$C_{50}H_{87}N_7O_{13}$	12.9	Surfactin A-CH_2
20.23	BZR 517	1008.6465	1008.6591	$[M + H]^+$	$C_{51}H_{89}N_7O_{13}$	12.5	Surfactin A
21.88	BZR 517	1022.6619	1022.6748	$[M + H]^+$	$C_{52}H_{91}N_7O_{13}$	12.6	Surfactin B
22.83	BZR 517	1036.6783	1036.6904	$[M + H]^+$	$C_{53}H_{92}N_7O_{13}$	11.7	Surfactin C

Compounds such as surfactin and their isomers and homologues were found in both samples. Mass chromatograms and mass spectra of the detected compounds are shown in Figures 9–12, respectively.

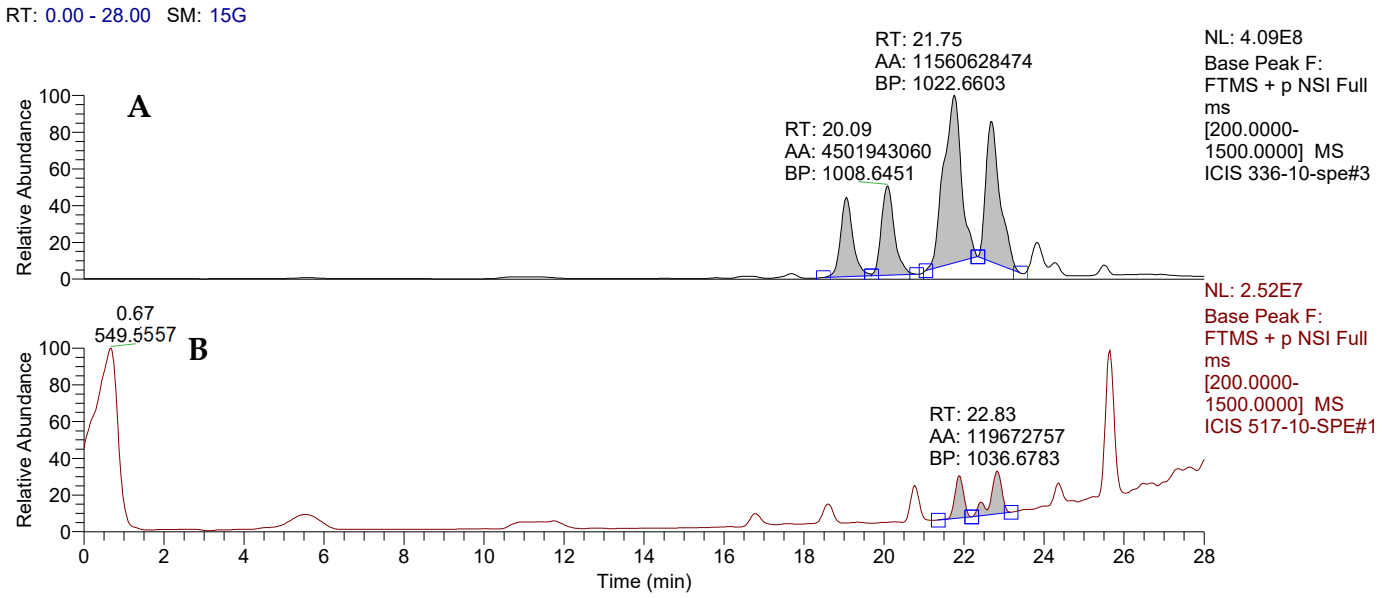


Figure 9. Total ion mass chromatogram in positive ion detection mode for samples BZR 336 g (**A**) and BZR 517 (**B**).

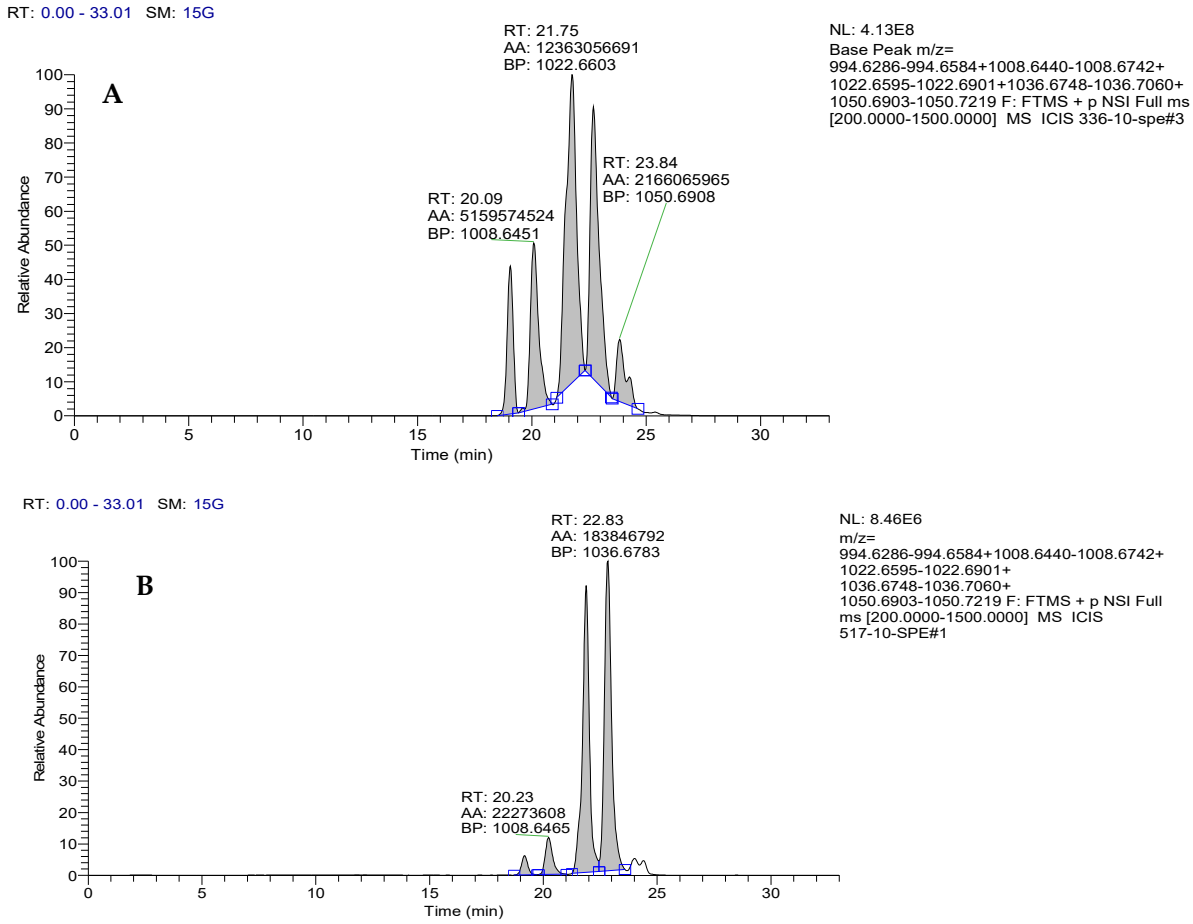


Figure 10. Base peak mass chromatogram in positive ion detection mode of Surfactin A-CH2, Surfactin A, Surfactin B, Surfactin C, and Surfactin C + CH_2 for samples BZR 336 g (**A**) and BZR 517 (**B**).

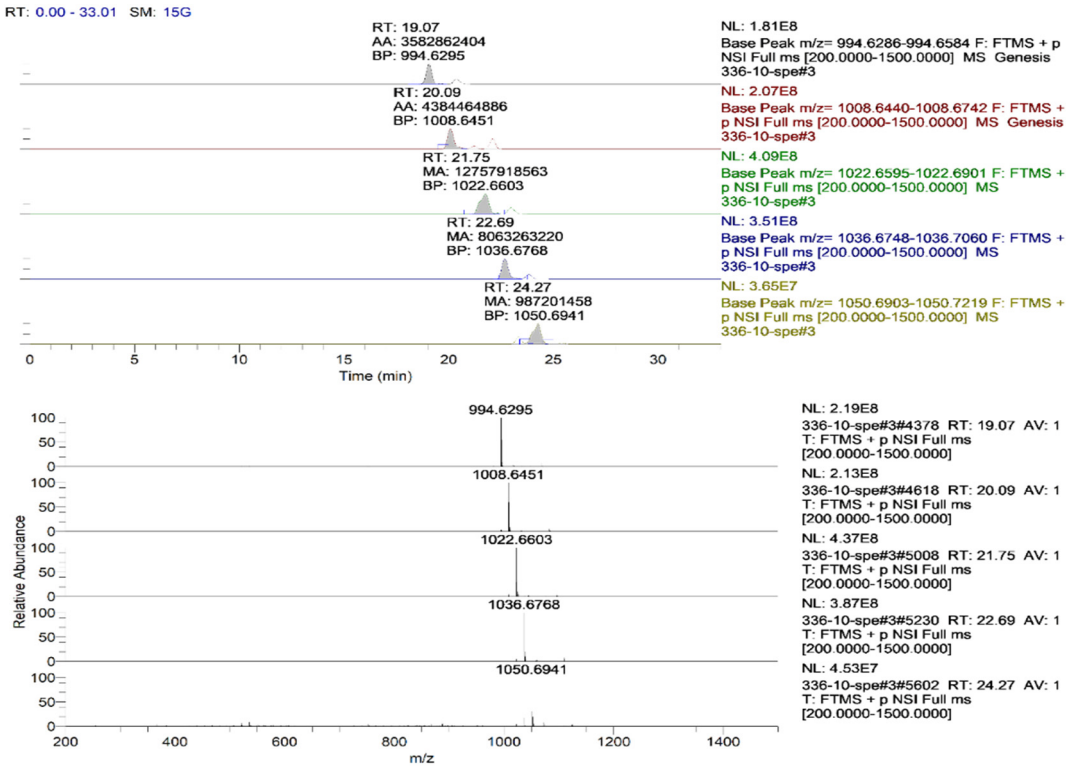


Figure 11. HPLC-ESI+-HRMS spectra of Surfactin A-CH_2, Surfactin A, Surfactin B, Surfactin C, and Surfactin C + CH_2 for sample BZR 336 g.

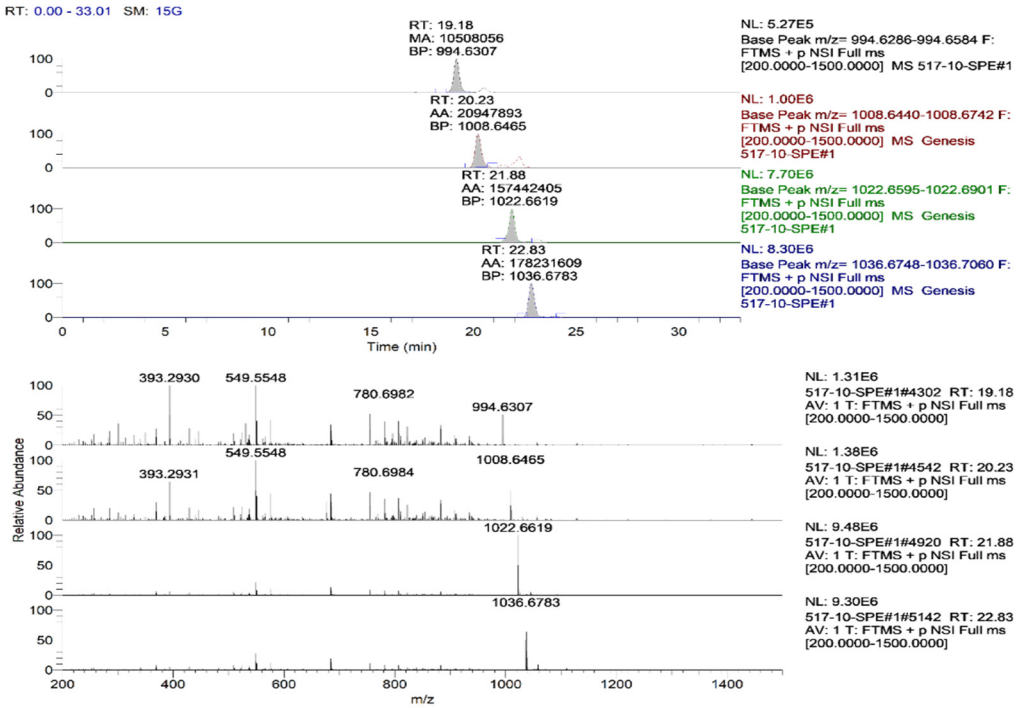


Figure 12. HPLC-ESI+-HRMS spectra of Surfactin A-CH_2, Surfactin A, Surfactin B, and Surfactin C for sample BZR 517.

During the data analysis, significant differences in areas were found for Surfactin A-CH_2 (340 times), Surfactin A (20 times), Surfactin B (81 times), and Surfactin C (45 times) in sample BZR 336 g (Figure 11) compared to sample 517 (Figure 12). Thus, it can be assumed

that strain BZR 517 produces fewer metabolites than bacterial strain BZR 336 g. Identified compounds are presented in Table 1, their mass spectra are presented in Figures 11 and 12, and the structures of Surfactin A, Surfactin B, and Surfactin C are presented in Supplementary Data. Chromato–mass spectrometric parameters of the detected surfactin and homologues (*B. velezensis* BZR 336 g and *B. velezensis* BZR 517) are presented in Table 1.

3.2. Climatic Chamber Research

The efficacy of a biological agent is largely determined by the ability to protect seeds and seedlings. Testing on an artificial infectious background provides an objective efficacy assessment of strains.

In the control, the development of the disease was 48.2%. This indicated a sufficient infectious background for an objective assessment. BE of the chemical reference was 25.5%. The maximum efficiency was shown by the biological reference to be 39.6% (Table 2). Notably, the strains had a positive effect on seed germination.

Table 2. Biological efficacy (BE, %) of *B. velezensis* BZR 336 g and *B. velezensis* BZR 517 against *F. graminearum* BZR 4 on winter wheat in a climatic chamber.

Treatment	Germination, %	Development, %	BE, %
Control	87.6 ^{a}	48.2 ^{b}	-
Chemical standard Kinto Duo, SC	92.3 ^{a}	35.9 ^{ab}	25.5
Biological standard Fitosporin-M, L	91 ^{a}	29.1 ^{ab}	39.6
B. velezensis BZR 336 g	94.3 ^{a}	37.3 ^{ab}	22.5
B. velezensis BZR 517	95.7 ^{a}	32.6 ^{a}	32.3

Note: Different letters indicate significant differences ($p < 0.05$) according to Duncan's test.

BE of the studied strains was higher or close to the chemical reference. The efficiency of BZR 336 g was 22.5% and that of BZR 517 was 32.3%.

The efficiency of the strains rarely exceeded the efficiency of the references. However, the strains had a good effect on germination. Germination with BZR 336 g was 94.3% and that with BZR 517 was 95.7%. This is 2–4.7% higher compared to the references (Table 1). Presumably, BZR 336 g and BZR 517 do not simply provide protection against diseases but also reduce the stress load on plants.

3.3. Field Research

We tested the *B. velezensis* BZR 336 g and *B. velezensis* BZR 517 strains on winter wheat under conditions of stationary crop rotation from 2012 to 2015 at the FRCBPP experimental base.

The efficiency of *B. velezensis* BZR 336 g and BZR 517 significantly exceeded the chemical reference by 27.5–35% in 2012–2013. *B. velezensis* BZR 517 showed the highest efficiency. The yield was 7.9 t/ha when *B. velezensis* BZR 336 g and BZR 517 were applied, which remarkably exceeded both the chemical reference and control (Table 3).

The efficiency of strains was slightly lower than that of chemical and biological references—17.2–22.8% in 2013–2014. The efficiency of chemical and biological references was 17.4 and 26.2%, respectively. The yield when using the strains was 7.2–7.6 t/ha, which exceeds the control (4.0 t/ha) and the chemical reference (7.0 t/ha) and is close to the biological reference (7.4 t/ha).

In 2014–2015, there was a shift in sowing dates due to adverse weather conditions. The BE was below the reference efficiency—13.6%. The efficiency of the biological reference was 16.0%. The chemical reference was 25.9% effective. Yields exceeding the control were obtained using the chemical reference 8.3 t/ha and *B. velezensis* BZR 336 g using 7.9 t/ha.

Research results prove that seed treatment with liquid culture of the studied strains protects winter wheat and provides an additional yield sometimes higher than the biological

and chemical references. This, in turn, contributes to the range expansion of microbial biological products in the segment of the Russian market of environmentally friendly plant protection products.

Table 3. Biological efficacy (BE, %) and yield of winter wheat of Kalym cultivar treated with BZR 336 g and BZR 517 under stationary crop rotation, 2012–2015.

Experimental Options	Development, %			Biological Efficacy against *Fusarium* Root Rots, %, by Years			Productivity, t/ha		
	2012–2013	2013–2014	2014–2015	2012–2013	2013–2014	2014–2015	2012–2013	2013–2014	2014–2015
Control	22.2 ^{b}	36.1 ^{b}	25.3 ^{b}	-	-	-	4.0 ^{b}	6.9 ^{a}	7.7 ^{a}
Chemical standard	20.0 ^{b}	26.7 ^{a}	18.8 ^{a}	10.0	26.2	25.9	6.9 ^{c}	7.0 ^{a}	8.3 ^{b}
Biological standard	–	29.8 ^{a}	21.3 ^{ab}	–	17.4	16.0	–	7.4 ^{c}	7.5 ^{a}
B. velezensis BZR 336 g	13.9 ^{a}	27.9 ^{a}	21.9 ^{ab}	37.5	22.8	13.6	7.9 ^{a}	7.2 ^{b}	7.9 ^{ab}
B. velezensis BZR 517	12.9 ^{a}	29.9 ^{a}	26.3 ^{b}	45.0	17.2	0	7.9 ^{a}	7.6 ^{d}	7.5 ^{a}

Note: Different letters indicate significant differences ($p < 0.05$) according to Duncan's test.

4. Discussion

Bacillus species are widely used for biological control of plant diseases, which has been repeatedly reported [1,3,10,13,15–18,20,21]. Biological control is a promising plant pathogen control strategy.

It is reported that Bacillus has the ability to produce many antimicrobial substances [13]. These bacteria can inhibit the growth of pathogens and spore germination [17] and cause deformation damage to mycelial hyphae [14]. Understanding the mode of action between bioagents on cereal pathogens is important for the use of *Bacillus* as a reliable biocontrol agent.

We used two initial strains, *B. velezensis* BZR 517 and BZR 336 g, isolated from the rhizosphere of winter wheat in Krasnodar (Kuban Region, Krylovsk district 46°19′19′′ N 39°57′51′′ E; Pavlovsky district 46°08′22′′ N 39°47′19′′ E). As research has shown, these strains were the most promising among other strains of the Bioresource Collection "State Collection of Entomoacariphages and Microorganisms". Individual cultivation conditions (temperature, acidity of the environment, and incubation time) were selected for each of the studied strains, in order to maximize the production of antifungal metabolites by each strain [27].

We selected the fungus *F. graminearum*, as it is the most common and aggressive crop pathogen worldwide [4]. In addition, *F. graminearum* causes significant economic damage to crops in the south of Russia.

In our study, the strains had a fungicidal and fungistatic effect on *F. graminearum* BZR 4 based on the results of double culture microscopy. We observed various signs of pathogen mycelium degradation during coincubation with *B. velezensis* BZR 517 and BZR 336 g—curvature, swelling, and rupture (Figures 3 and 4). Bacterial chemotaxis towards the hyphae and hyphae encirclement by bacterial cells (Figure 5b) were recorded. In some cases, bacteria colonized the hyphae, after which the latter was destroyed (Figure 5c).

During the experiment, we recorded two fundamentally different types of hyphae damage: (1) Gradual dissolution without shape loss, where the hypha remains long and even but becomes transparent and dissolves over time (Figure 7a). This type of damage is observed when the contents of the hypha leak from its apical compartment or when the hypha is dissolved by bacteria adsorbed on it. (2) Change in hyphae shape, which was expressed as shortening, swelling, and increased branching, where the hypha remains pigmented but shortens and thickens (Figure 7b). We assume a violation of the integrity of the cell wall or cytoplasmic membrane to be the second type of damage. As a result, the balance of ions inside and outside the cell is disturbed. The antifungal effect of BZR 517 is somewhat less pronounced and manifests itself mainly remotely by diffusion of antifungal metabolites into the cultivation medium. The antifungal effect of BZR 336 g

metabolites is more pronounced. It is more aggressive towards *F. graminearum* and exhibits active chemotaxis towards hyphae using them as a food source.

Results obtained by the method of bioautography suggest that the strains BZR 336 g and BZR 517 produce iturin A and surfactin, which together may have synergistic effects (Figure 8). It is reported that iturin displayed strong fungicidal activity and causes severe morphological changes in conidia and significant distortions of *F. graminearum* hyphae. In addition, iturin A caused leakage and/or inactivation of the cellular contents of *F. graminearum* [4].

The role of bacterial metabolites of *B. subtilis* in the control of plant pathogens and especially *F. graminearum* has been repeatedly pointed out in the scientific literature. Gong A.D. et al. [3] reported a bacterial strain of *B. amyloliquefaciens* S76-3 that exhibited potent antifungal activity against *F. graminearum*. HPLC analyses showed that S76-3 produces three classes of cyclic lipopeptides, including iturin, plipastatin, and surfactin. Major morphological changes in conidia and significant distortions in *F. graminearum* hyphae occurred under the influence of these compounds. The researchers observed vacuolization and leakage of hyphae cellular contents, fracture of cell walls and plasma membranes, and distortion of *F. graminearum* hyphae.

Zhao Y. et al. [17] report a strain of *B. subtilis* SG6 that significantly inhibits the growth and sporulation of *F. graminearum* mycelium. In the presence of this antagonist, there is exfoliation of the surface of pathogen hyphae, as well as degradation of cell walls and degeneration of cell organelles. Researchers attribute the antifungal activity of *B. subtilis* SG6 to the production of fengycins and surfactins.

In J.M. Palazzini et al. [15], the *B. subtilis* RC 218 strain was demonstrated as a biocontrol agent for *F. graminearum*. It is reported that the antifungal activity of the strain may be associated with the production of lipopeptides from the surfactin, fengycin, and iturin families.

The use of HPLC-HRMS made it possible to detect and identify lipopeptides. In this paper, we used nano LC and high-resolution mass spectrometry, so it was possible to detect and identify surfactin and its homologues in biological samples. The shown homology profile of surfactin can serve as an excellent tool in bioanalytical applications to control the resulting bioactive metabolites and thus be useful in targeted metabolomics. In the future, it is planned to conduct quantitative non-targeted metabolomics using oxygen exchange ($^{16}O/^{18}O$) for more reliable identification by high-resolution mass spectrometry of the Orbitrap type. Moreover, it is planned to search for known compounds in such databases as mzCloud, HMDB, GNPS, etc., and apply Python programming to predict chemical structures, retention times, and other chromatographic and mass spectrometric parameters.

Only compounds of surfactin and their isomers and homologues were found in samples BZR 336 g and BZR 517 (Figures 9–12). *Bacillus* spp., with proven colonization aptitude, have been well studied and are frequent candidates for use as biocontrol agents. *Bacillus* spp. are effective as biocontrol agents against plant pathogens due primarily to their production of various cyclic lipopeptides [17]. It is known that bacterial lipopeptides can damage plasma membranes of hyphae, resulting in cell death [3,17].

Evidently, *B. velezensis* BZR 336 g accumulates more active metabolites than *B. velezensis* BZR 517. This is supported by visual evaluation of chromatographic plates, data received from HPLC-HRMS, and higher BE. Although both strains belong to the same *B. velezensis* species, clearly each of them produces its own individual metabolite complex, which differs in composition and action spectrum.

The efficacy of a biological agent is largely determined by the ability to protect seeds and seedlings. Testing on an artificial infectious background provides an objective efficacy assessment of strains. In this environment, the protective properties of the bioagent are most fully manifested. Moreover, here we obtain a guaranteed infection and manifestation of the disease with a controlled infectious load.

An experiment on an artificial infectious background in a climatic chamber illustrated the antifungal effect of strains on living objects. BE of *B. velezensis* BZR 336 g and BZR 517 was close to that of the references. In addition, judging by the germination rates, the strains

are likely to reduce the stress load on plants, which is important for a biocontrol agent. Altogether, our research in the field conditions has confirmed that the strains developed a strong protective role against *Fusarium* root rots. The protective effect varied depending on weather conditions, development stage, distribution, and sowing time.

We outlined in our previous article [27] that *B. velezensis* BZR 336 g and BZR 517 synthesized significant amounts of other fungitoxic metabolites. We see great potential for future research here. The *B. velezensis* strains BZR 336 g and BZR 517 showed antagonistic activity against *P. tritici-repentis* in laboratory and field tests [30]. Moreover, we noted the effectiveness of strains for the biocontrol of currant Septoria leaf spot [31], raspberry spur blight, and potato rhizoctonia rot [32] in the adverse climate of Western Siberia. We expect that further studies of these bioagents will contribute to their successful commercialization. The appearance on the market of new biological plant protection products against a wide range of pathogens will promote the development of organic farming.

5. Conclusions

B. velezensis BZR 336 g and BZR 517 inhibit the growth of the fungal pathogen *F. graminearum* through deformation and lysis of hyphae. We detected the cyclic lipopeptides iturin A and surfactin in extracts of this bacteria by bioautography and assume they are responsible for the observed effect on *F. graminearum*. Studies on an artificial infectious background of infection with *F. graminearum* BZR 4 indicate that bioagents not only inhibit the pathogen but also stimulate the immune status of the plant itself. Bioagents *B. velezensis* BZR 336 g and BZR 517 showed an efficacy of up to 45.0% against pathogens, with a yield of up to 7.9 t/ha. The use of *B. velezensis* BZR 336 g and BZR 517 for biocontrol of *F. graminearum* in winter wheat provides an environmentally friendly approach for sustainable agriculture.

6. Patents

Asaturova A.M., Tomashevich N.S., Zhevnova N.A., Homyak A.I., Dubyaga V.M., Pavlova M.D., Kozitsyn A.E., Sidorova T.M. "Biofungicide for protecting crops from diseases and increasing yields"/patent for invention RUS 2621356 03.12.2015.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12081956/s1, Table S1: Structures of Surfactin A, Surfactin B, and Surfactin C.

Author Contributions: Conceptualization, A.M.A., N.A.Z., N.S.T. and Y.I.K.; methodology, A.M.A., N.A.Z., N.S.T., Y.I.K. and A.I.H.; software, A.P.Z. and B.S.T.; validation, A.M.A.; formal analysis, N.A.Z. and N.S.T.; investigation, A.M.A., N.A.Z., N.S.T., V.M.D. and Y.I.K.; resources, A.M.A.; data curation, A.M.A., N.A.Z. and N.S.T.; writing—original draft preparation, N.A.Z., T.M.S., B.S.T. and A.P.Z.; writing—review and editing, N.A.Z., N.S.T., A.M.A., T.M.S., V.M.D. and A.I.H.; visualization, N.A.Z. and A.P.Z.; supervision, V.D.N.; project administration, A.M.A. and N.S.T.; funding acquisition, A.M.A. All authors have read and agreed to the published version of the manuscript.

Funding: The research was carried out in accordance with the State Assignment of the Ministry of Science and Higher Education of the Russian Federation within the framework of research on the topic no. FGRN-2022-0003 «Study of biodiversity and bioresources of useful biota, ensuring the effectiveness of mechanisms of natural biocenotic regulation, and replenishment of bioresource collections».

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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