Biological Control Ability and Antifungal Activities of Bacillus velezensis Bv S3 against Fusarium oxysporum That Causes Rice Seedling Blight

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Abstract: Fusarium oxysporum, a primary soil-borne fungus that affects rice seedlings globally, is responsible for rice seedling blight (RSB), which reduces seedling quality and survival rates. The synthetic fungicides used to treat this disease negatively affect human health and the environment. A biocontrol bacterial isolate, Bacillus velezensis Bv S3, isolated from the rice rhizosphere, showed a strong antagonistic effect on RSB-causing F. oxysporum. The ratio of the longest to the shortest radius of F. oxysporum following Bv S3 inoculation was 2.52 (cm/cm) in a plate standoff experiment. This was different from the other biocontrol strains. Bv S3 exhibits a wide spectrum of antifungal activity against various pathogenic fungi that cause RSB. When 10% Bv S3 liquid culture filtrate was applied, it dramatically reduced F. oxysporum spore germination and mycelial growth, with inhibition rates of 66.7%, and 45.7%, respectively, and caused hyphal malformations. Furthermore, the Bv S3 suspension (1 × 10⁸ CFU/mL) reduced RSB by 65.5% and 76.5% in pot experiments, effectively promoted the growth of rice seedlings, and improved the activities of neutral phosphatase, urease, invertase, and catalase in rice rhizosphere soil. The active substances produced by Bv S3 were sensitive to temperature and ultraviolet irradiation, and the antifungal effect significantly increased after 90 min of exposure, with antifungal effect observed at pH 7. Bv. S3 effectively reduced the incidence of RSB and showed potential as a biocontrol agent.

Keywords: rice seedling blight; Bacillus velezensis; antifungal activities; biological control; soil enzyme activity

1. Introduction

Rice (Oryza sativa) is an important cereal crop that provides energy for most of the world’s population [1]. Currently, rice is cultivated in more than 100 countries [2]. According to several studies, the area used for rice cultivation in northeastern China has expanded by 3.68 million hectares over the past 20 years, and Chinese rice production has increased considerably [3–6]. Rice seedling blight (RSB), a common rice seedling disease, poses a significant threat to global rice production, particularly in the cold regions of northern China [7]. RSB has been reported in Asia, Europe, Africa, and the Americas [7]. Rice yield loss due to RSB can range from 8% to 50%, severely limiting rice quality and yield [8]. The typical symptoms of RSB include withered tips, chlorosis, stunting, leaf loss, inhibition of root growth, and crown rot. Dark brown necrotic lesions are observed on the roots or mesocotyls. In severe cases, hyphae are attached to the diseased parts, causing the rice seedlings to die [9,10].

RSB is a serious soil-borne disease observed worldwide and caused by multiple pathogens, including Fusarium oxysporum [4,11], F. tricinctum [12], Curvularia lunata [13],...
Avoiding excess moisture in the seedbed, planting resistant varieties, and using fungicides are common approaches to reduce RSB [24,25]. The primary strategy for managing RSB is the application of synthetic fungicides. Common agricultural fungicides used to control \textit{Fusarium} spp. include imazalil, tolclofos-methyl, fenaminosulf, and hymexazol [26–29]. However, with the emergence of problems such as residues of synthetic fungicides, ecological destruction, and fungicide resistance of pathogens, researchers are searching for other ecologically friendly control strategies for RSB [30].

Biological control is a potential technique for managing various plant diseases [31] and has attracted attention as an eco-friendly method for controlling RSB using beneficial microorganisms [23], including \textit{Bacillus velezensis} [23], \textit{Paenibacillus terrae} [11], \textit{Bacillus cereus}, \textit{Actinomycetes} [32], and \textit{Paenibacillus alvei} [33]. Currently, only a few antagonistic microorganisms can effectively control RSB. Therefore, a primary task in RSB control research is to explore new antagonistic bacterial resources and elucidate their mechanisms of action [11].

\textit{Bacillus} spp. are widespread in natural habitats, including soil and plants [31]. As biological control agents, \textit{Bacillus} spp. produce active secondary metabolites that may promote the healthy growth of plants via various mechanisms such as direct antibiosis, suppression of several soil-borne plant pathogens, plant growth promotion, and induction of systemic resistance in plant hosts [31,34]. Fan et al. showed that \textit{Bacillus velezensis} FZB42 was able to promote the growth of plants [35]; \textit{Bacillus subtilis/amyloliquefaciens} can produce antibiotics that directly suppress several soil-borne plant pathogens, according to a study by Cawoy, H. et al. [36]. \textit{Bacillus amyloliquefaciens} Ag1 was demonstrated by Dihazi, A. et al. to enhance systemic resistance in date palm, promote phenolic production, and raise peroxidase (POX) activity in date palm roots [37]. Moreover, certain \textit{Bacillus} sp. strains have strong colonising capabilities, can be easily handled, fight pathogens for niche or nutritional requirements, and directly generate active secondary metabolites, such as iturin, surfactin, and fengycin, on numerous plant pathogens [38]. Thus, \textit{Bacillus} spp. were screened as excellent biocontrol agents for RSB in this study.

The objectives of this study were as follows: microorganisms with the ability to control RSB were screened and identified; then, the antifungal mechanisms and stability of the antifungal substances of the biocontrol organisms were analysed (pH, temperature, and UV radiation); finally, the impact of the biocontrol bacteria on soil enzyme activity was determined, and the effectiveness of using potential biocontrol bacteria for the treatment of RSB was evaluated.

2. Materials and Methods

2.1. Isolation of Bacterial Isolates

In total, 156 bacterial isolates were isolated from the rhizosphere soil of rice seedlings with RSB in the rice planting area of Harbin, China, with the method of soil dilution [39]. The sample location was the Xiangyang planting base of Northeast Agricultural University, the rice variety was “Longdao 203”, and the sampling depth was between 20 and 30 cm. Each of the five sampled plots contained 1500 g of soil. Individual colonies were isolated and purified after 72 h of incubation at 28 °C on beef extract peptone medium (BPM) (Beijing Aoboxing Biology Technology Co., Ltd., Beijing China).

2.2. Screening of Biological Control Bacteria against RSB

\textit{Fusarium oxysporum} (MT180464), which was isolated and stored by our team and is the dominant RSB-causing pathogen in Northeast China, accounting for 48% of the total
number of fungi isolated, was selected as the target pathogen [4]. The confrontation culture method was used to evaluate the antagonistic effects of the screened bacteria [40]. After 48 h of activation of the isolated bacterial isolates on BPM plates at 28 °C, each bacterial strain was inoculated at a distance of 3 cm from the centre of the potato dextrose agar (PDA) (pH = 5.6 ± 0.2) (Beijing Aoboxing Biology Technology Co., Ltd., Beijing, China) of the plate with PDA by the scribing method. A 0.7-cm-diameter colony of *F. oxysporum* was inoculated at the centre of the PDA plate and cultured at 26 °C for 6 d. The maximum and minimum radii of the *F. oxysporum* colonies were measured [40]. Strains with the largest longest-to-shortest radius ratios were selected for further in-depth analysis.

2.3. Reduction of RSB by Biocontrol Bacterial Suspensions

Bacteria with strong antagonistic effects were preliminarily screened in pots to obtain the target biocontrol bacterial isolates (named Bv S3), and the control effect was subsequently determined. The S3 biocontrol bacteria isolates were inoculated in liquid BPM, propagated for 48 h, and diluted to 1 × 10^8 CFU/mL (which corresponded to an optical density (OD) of 0.1 measured on a spectrophotometer (Anolen Beijing Biotechnology Co., Ltd., Beijing, China) at 600 nm). *F. oxysporum* was grown on PDA medium at 26 °C for 5 d, washed with sterile water, filtered with sterile double-layer gauze, and diluted to a concentration of 1 × 10^6 spores/mL using a haemocytometer.

The efficacy of Bv S3 in suppressing RSB caused by *F. oxysporum* was investigated under greenhouse conditions (23 ± 3 °C, 75% relative humidity). Healthy, plump rice plants (cv. Daohuaxiang-2) were sterilised in 75% alcohol for 1 min and then washed with sterile water. Ten seeds were planted in a pot (10 × 10 cm) on top of potting soil (vermiculite/soil ratio of 1:2) and covered with sterile soil. Four treatments were set as follows: (i) as a control, 5 mL of sterile water; (ii) 3 mL conidia suspension (1 × 10^6 spores/mL) of *F. oxysporum* without the biocontrol bacteria; (iii) 3 mL conidia suspension (1 × 10^6 spores/mL) of *F. oxysporum* and 5 mL suspension of the biocontrol bacteria Bv S3 containing 1 × 10^8 CFU/mL; (iv) 3 mL conidia suspension (1 × 10^6 spores/mL) of *F. oxysporum* and 3 mL of 45% prochloraz (Sportak, with an effective concentration of 100 µg/mL) (United Bio-Shanghai and Shanghai Pharmaceutical Xiayi Co., Ltd., Shanghai, China); and (v) 5 mL suspension of the biocontrol bacteria Bv S3 containing 1 × 10^8 CFU/mL. The experiment was conducted twice under the same conditions, with three replicates per treatment. A 5 mL suspension (1 × 10^8 CFU/mL) of Bv S3 was placed into each pot on the planting day with the same amount of 0.9% normal saline as the control. After 24 h, 3 mL of conidial suspension (1 × 10^6 spores/mL) of *F. oxysporum* was added to each pot. Rice seeds were soaked in 45% prochloraz for 30 min, dried, and planted in culture pots. After 24 h, 3 mL of a conidial suspension (1 × 10^6 spores/mL) of *F. oxysporum* was added to each pot. After 7 d, the same Bv S3 bacterial suspension amount was inoculated into the corresponding culture bowl. The disease severity of rice was investigated 28 d after sowing, and the percentage reduction in the treatment compared to the control was calculated.

Based on the disease severity of rice seedlings, disease severity was graded visually using a scale from 0 to 4, defined as follows [11]: 0 = no symptoms; 1 = a few small lesions accounting for ≤25% of the stem, or rice leaves begin to turn yellow (however, no visible lesions appear); 2 = large lesions accounting for 25 to ≤50% of the stem, or rice leaves are yellowish, lobus cardiacus are curled, and the sick plants are dwarved; 3 = large lesions accounting for 50 to ≤75% of the stem, or rice leaves visibly dried up, stem base turns brown, and plants stop growing; and 4 = lesions accounting for >75% of the stem, or rice leaves are dark brown, soft, and rotting, and can be easily uprooted when seedlings are pulled by hand.

The disease index (DI) was calculated as Σ (number of diseased rice seedlings at each scale × relative grade)/(total number of surveyed plants × highest disease rate) × 100.

Fresh weight: after removal from the pots, the rice was cleaned with distilled water, excess water was drained, and the fresh weight was measured with a balance. Plant height: the height of the rice was determined by keeping the straight edge vertically pressed against
the base of the rice. Root length: following removal from the soil and thorough cleaning, the roots of the rice plants were straightened and measured using a straight edge.

2.4. Micro-Morphological Observation of Biocontrol Bacteria Bv S3

The morphological characteristics of Bv S3 were observed on nutrient agar (NA) using a scanning electron microscope (SU8010; Hitachi, Tokyo, Japan).

The following steps were carried out to prepare a sample for scanning electron microscopy: the bacterium was activated on BPM medium by adding glutaraldehyde for 1.5 h. The treated samples were washed several times with phosphate-buffered saline (PBS), dehydrated with ethanol, and dried in a freeze-dryer (ES-2030; Hitachi, Tokyo, Japan). Finally, the sample was fixed to the sample table of the scanning electron microscope with conductive tape and covered with a metal film for further inspection.

2.5. Identification of Biocontrol Bacteria Bv S3

The physiological and biochemical traits of Bv S3 were measured according to previous reports by Schaad et al. [41], Buchanan [42], and Dong et al. [43]. Physiological and biochemical traits were assessed using the following indicators: Gram staining; catalase test; hydrolysis of casein, starch, gelatine, and sulphur amino acids; nitrate reduction; the V-P and MR tests; and the utilisation of glucose, mannitol, fructose, sucrose, lactose, cellobiose, arabinose, xylose, maltose, rhamnose, citrate, sorbitol, inositol, and malonate using physiological and biochemical traits kits.

Genomic DNA of Bv S3 was extracted using a plant genomic DNA extraction kit (Beijing ComWin Biotech Co., Ltd., Beijing, China). The amplification and sequencing of 16S rRNA were performed using the general primers: 27F (5′-AGAGTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplification experiments were performed using a PCR mix kit (CW0556, Beijing ComWin Biotech Co., Ltd., Beijing, China) in a total volume of 50 µL [36]. The PCR program was carried out as follows: 5 min at 94 °C; 36 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1.5 min at 72 °C; and a final extension step of 10 min at 72 °C. The amplification and sequencing of gyrB were performed using the specific primers: 22F (5′-GAAGTTATCATGACGGTACTTC-3′) and 1240R (5′-AGCGTACGAAATGTGAGAACC-3′). The PCR program was carried out as follows: 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C; and a final extension step of 5 min at 72 °C [44]. The amplified product was sequenced by Genewiz Biotechnology Co., Ltd. (Suzhou, China) and analysed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 29 August 2023). Phylogenetic trees of Bv S3 were constructed using the PhyloSuite software 1.2.2 based on the Bayesian inference (BI) method [45].

2.6. Antifungal Spectrum

The antagonistic effect of the biocontrol bacteria Bv S3 was determined against eight fungal pathogens of major field crop diseases; among them, *F. incarnatum* (OQ644654), *F. redolens* (MT182983), *F. equiseti* (MT180475), *F. solani* (MT180477), and *F. tricinctum* (MT180474) were the pathogenic fungi causing RSB, and *Chaetomium globosum* (ON204053), *F. proliferatum* (ON204050), and *F. petroliphilum* (OQ644655) were the pathogenic fungi causing root rot of soybean (RRS). Pathogenic fungi were collected at the Plant Pathology Laboratory of the Northeast Agricultural University, China. The measurement and evaluation methods are the same as those described in the Section 2.

2.7. Effects of Bv S3 Filtrate on Conidial Germination, Mycelial Growth, and Morphology of *F. oxysporum*

A 0.5 mL suspension of biocontrol bacteria Bv S3 activated at 28 °C and 180 rpm for 24 h was inoculated into liquid BPM under the same conditions for 7 d. The Bv S3 aseptic filtrate was produced using a sterilised bacterial filter (142 mm, YY3014236, Millipore, Burlington, MA, USA). The absence of turbidity in the filtrate indicated that it was sterile when left at 25 °C for 48 h.
An aseptic filtrate of Bv S3 was added to the PDA medium at a final concentration of 1%, 5%, and 10%. Equivalent amounts of liquid BPM were added to the PDA medium as a control. Each treatment consisted of three replicates, and the experiment was conducted twice. A mycelial plug (7 mm in diameter) of *F. oxysporum* was placed at the centre of the PDA plate for 96 h at 26 °C. The inhibition rate of fungal was calculated.

The conidia and hyphal suspensions of *F. oxysporum* scraped from the corresponding treatments were filtered with a double-layer sterile gauze, and the conidial suspension was adjusted to $1 \times 10^8$ spores/mL using a haemocytometer and then cultivated in a cell culture plate. Each treatment consisted of three replicates. For each treatment, the sample was incubated at 26 °C until the conidia of the control treatment germinated to 60%, and the number of conidia in each treatment was counted. The number of germinated conidia (100 spores per treatment) was determined under a light microscope (Leica Microsystems CMS GmbH; Leica Microsystems, Wetzlar, Germany). The rate of inhibition of conidial germination was calculated.

A mycelial plug (7 mm in diameter) of *F. oxysporum* was incubated at 26 °C for 12 h. Fresh hyphae were scraped and submerged in Bv S3 aseptic filtrate at concentrations of 1%, 5%, and 10%. The hyphal morphology was observed and compared under a light microscope after 14 h.

### 2.8. Stability of Antifungal Substances

An aseptic filtrate of Bv S3 at a concentration of 5% was incubated in a water bath at 20, 40, 60, 80, and 100 °C and autoclaved (121 °C) for 20 min. Then, the impact of aseptic filtrates treated with different temperatures on the mycelial growth of *F. oxysporum* was evaluated [46]. Following a 7 d incubation period at room temperature, the mycelial diameters of the different treatment groups were measured, and the mycelial growth rate method was used to calculate the inhibition rate. A smaller mycelium diameter indicated greater antifungal activity of the aseptic filtrate.

The pH of the aseptic filtrate of Bv S3 was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. The aforementioned method was employed to determine the impact of different pH treatments on Bv S3.

The aseptic Bv S3 filtrate was exposed to UV light (100 J/s/cm$^2$) at a distance of 1 cm for 30, 60, and 90 min. The effects on Bv S3 were measured using the aforementioned method.

### 2.9. Effect of the Strain on Soil Enzyme Activity

Each treatment was performed in the same manner as mentioned in the Section 2. Soil samples were obtained from the rice rhizosphere every seven days and five consecutive times after the first inoculation of Bv S3. Soil samples (10 g) from five points were collected each time and stored immediately in an ultra freezer at –80 °C for testing after the last sampling. Enzyme activities were determined using soil enzyme activity kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) which included soil urease (S-UE), neutral phosphatase (S-NP), invertase (S-SC), cellulase (S-CL), and catalase (S-CAT), according to the manufacturer’s instructions for specific operation methods.

### 2.10. Data Analysis

One-way analysis of variance (ANOVA) was conducted using SPSS (version 17.0; IBM/SPSS, Armonk, NY, USA). Duncan’s multiple range test revealed a significant difference between the treatment means at $p < 0.05$. ANOVA data are provided in the Supplementary Materials.

### 3. Results

#### 3.1. Isolation and Screening of Biological Control Bacteria

All 156 strains isolated from rice rhizosphere soil were tested for their antagonistic effects against *F. oxysporum*. Seven strains had better antagonistic effects than the other
strains, and Strain 3 (named Bv S3) showed the best antagonistic effect (Table 1), which showed good potential for biocontrol, with an average ratio of 2.52. Preliminary screening of the potted cultivated plants showed that Bv S3 had the best control effect on RSB. Bv S3 was chosen for subsequent experiments. Additionally, Bv S3 showed strong antagonistic activity against pathogenic fungi associated with major field crop diseases (Table 2, Figure 1), including *F. solani* (RRS), *F. equiseti* (RRS), *F. redolens* (RRS), *F. incarnatum* (RRS), *F. tricinctum* (RRS), *Chaetomium globosum* (RRS), *F. proliferatum* (RRS), and *F. petroliphilum* (RRS).

**Table 1.** Antagonistic effect of seven biological bacteria strains on *Fusarium oxysporum* causing rice seedling blight.

<table>
<thead>
<tr>
<th>Biocontrol Strains No.</th>
<th>Maximum (cm)/Minimum (cm) Radius ± SE a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.65 ± 0.060b</td>
</tr>
<tr>
<td>2</td>
<td>1.87 ± 0.105b</td>
</tr>
<tr>
<td>3</td>
<td>2.52 ± 0.134a</td>
</tr>
<tr>
<td>4</td>
<td>2.26 ± 0.055a</td>
</tr>
<tr>
<td>5</td>
<td>2.33 ± 0.045a</td>
</tr>
<tr>
<td>6</td>
<td>1.84 ± 0.110b</td>
</tr>
<tr>
<td>7</td>
<td>1.64 ± 0.075b</td>
</tr>
</tbody>
</table>

a Values in the column indicate mean ± standard error (SE) of the maximum/minimum radius of the pathogens. Values followed by different letters are significantly different according to Duncan’s multiple range tests (*p* < 0.05). The df values, F-values, and *p*-values from the ANOVA of the data in Table 1 are displayed in the Supplementary Materials.

**Table 2.** Assessment of biological bacteria Bv S3 antagonistic activity against eight fungal pathogens in vitro.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Maximum (cm)/Minimum (cm) Radius ± SE a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium solani</em></td>
<td>1.996 ± 0.001c</td>
</tr>
<tr>
<td><em>F. equiseti</em></td>
<td>2.321 ± 0.002a</td>
</tr>
<tr>
<td><em>F. redolens</em></td>
<td>1.892 ± 0.001d</td>
</tr>
<tr>
<td><em>F. incarnatum</em></td>
<td>2.256 ± 0.002b</td>
</tr>
<tr>
<td><em>F. tricinctum</em></td>
<td>1.765 ± 0.001f</td>
</tr>
<tr>
<td><em>Chaetomium globosum</em></td>
<td>1.820 ± 0.002e</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>1.754 ± 0.004g</td>
</tr>
<tr>
<td><em>F. petroliphilum</em></td>
<td>1.568 ± 0.001h</td>
</tr>
</tbody>
</table>

a Values in the column indicate mean ± standard error (SE) of the maximum/minimum radius of the pathogens. Values followed by different letters are significantly different according to Duncan’s multiple range test (*p* < 0.05). The df values, F-values, and *p*-values from the ANOVA of the data in Table 2 are displayed in the Supplementary Materials.

**Figure 1.** Antagonistic effect of biological bacteria S3 on the mycelial growth of pathogenic fungi. (1) *Fusarium solani*, (2) *F. equiseti*, (3) *F. redolens*, (4) *F. incarnatum*, (5) *F. tricinctum*, (6) *Chaetomium globosum*, (7) *F. proliferatum*, (8) *F. petroliphilum*. 
3.2. Reduction of RSB following Application of Bv S3

As shown in Table 3, the disease index of rice seedlings inoculated with *F. oxysporum* alone was approximately 80. Compared with the control, the disease index of RSB was reduced dramatically by Bv S3; the efficacy was equivalent to that of synthetic fungicides, with that of Bv S3 being 65.5% and 76.5% and that of synthetic fungicides being 68.5% and 68.6% (Table 3, Figure 2). Compared to the endophytic biocontrol bacteria that Cao et al. [23] isolated from rice plants, Bv S3 was isolated from the rhizosphere soil of rice, which had advantages in antagonizing pathogenic fungi of rice seedlings in the soil, colonizing in the rhizosphere, and improving rice rhizosphere microecology. Moreover, Bv S3 significantly improved rice seedling growth, as evidenced by increased root length, plant height, and fresh weight.

Table 3. Efficacy of S3 bacterial suspension for reduction of rice seedling blight caused by *Fusarium oxysporum* in pot experiments.

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment</th>
<th>Application Dose</th>
<th>Fresh Weight (g)</th>
<th>Plant Height (cm)</th>
<th>Root Length (cm)</th>
<th>Disease Index (%)</th>
<th>Disease Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ii</td>
<td>--</td>
<td>0.09 ± 0.002b</td>
<td>7.7 ± 0.69c</td>
<td>5.6 ± 0.65b</td>
<td>74.2 ± 0.04a</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>5 mL/pot</td>
<td>0.12 ± 0.01a</td>
<td>12.1 ± 0.44a</td>
<td>8.7 ± 0.75a</td>
<td>25.6 ± 0.03b</td>
<td>65.5</td>
</tr>
<tr>
<td></td>
<td>iv</td>
<td>3 mL/pot</td>
<td>0.13 ± 0.004a</td>
<td>10.5 ± 1.32b</td>
<td>7.9 ± 0.51a</td>
<td>23.3 ± 0.10b</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>v</td>
<td>5 mL/pot</td>
<td>0.12 ± 0.005a</td>
<td>11.42 ± 0.38a</td>
<td>8.5 ± 0.88a</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>ii</td>
<td>--</td>
<td>0.09 ± 0.003b</td>
<td>6.9 ± 0.19b</td>
<td>5.6 ± 0.44c</td>
<td>87.5 ± 0.07a</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>5 mL/pot</td>
<td>0.12 ± 0.003a</td>
<td>11.0 ± 0.93a</td>
<td>8.7 ± 0.17a</td>
<td>20.6 ± 0.02b</td>
<td>76.5</td>
</tr>
<tr>
<td></td>
<td>iv</td>
<td>3 mL/pot</td>
<td>0.11 ± 0.01a</td>
<td>8.5 ± 0.14b</td>
<td>7.0 ± 0.34b</td>
<td>27.5 ± 0.01b</td>
<td>68.6</td>
</tr>
<tr>
<td></td>
<td>v</td>
<td>5 mL/pot</td>
<td>0.12 ± 0.01a</td>
<td>10.2 ± 0.12a</td>
<td>8.2 ± 0.17a</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Values in the column indicate mean ± standard error (SE) of two repeated experiments. Values followed by different letters were significantly different according to Duncan’s multiple range tests (p < 0.05). The letters used to indicate statistical differences must be read in the same column. ii: *F. oxysporum*; iii: *F. oxysporum* and Bv S3; iv: *F. oxysporum* and 45% prochloraz; v: Bv S3. The df values, F-values, and p-values from the ANOVA of the data in Table 3 are displayed in the Supplementary Materials.

3.3. Micro-Morphological Observation and Identification of Biocontrol Bacteria Bv S3

Bv S3 is a Gram-positive and rod-shaped bacterium, measuring 1.2 to 1.7 µm × 0.5 to 0.6 µm (n = 50), with round cell ends and frequently without a fold (Figure 3). Moreover, it is a catalase-producing anaerobic bacterium that can grow on glucose, mannitol, fructose, sucrose, lactose, and cellobiose and not on arabinose, xylose, maltose, rhamnose, citrate, sorbitol, inositol, or malonate. Additionally, it hydrolyses casein, starch, and gelatine and not sulphur-containing amino acids. The nitrate reduction and V-P tests were positive, and the methyl red test was negative. The 16S rDNA sequence of Bv S3 deposited in GenBank (accession no. MW786667) was 100% identical to that of *B. velezensis* SB1216 (accession no. CP015417.1). The gyrB sequence of Bv S3 deposited in GenBank (accession no. OR990557) was 100% identical to that of *B. velezensis* PD9 (accession no. MT338276.1). Phylogenetic tree analysis showed that Bv S3 and *B. velezensis* have close genetic relationships (red color in Figure 4) (The strains and accession numbers of the phylogenetic tree are detailed in the Supplementary Material). Therefore, Bv S3 was identified as *B. velezensis* based on the morphological and molecular phylogenetic analyses.
Figure 2. Biocontrol effects of *Bacillus velezensis* S3 suspension on rice seedling blight caused by *Fusarium oxysporum*. (i) 5 mL of sterile water; (ii) 3 mL conidia suspension \((1 \times 10^6 \text{ spores/mL})\) of *F. oxysporum* without biocontrol bacteria; (iii) 3 mL conidia suspension \((1 \times 10^6 \text{ spores/mL})\) of *F. oxysporum* and 5 mL suspension \((1 \times 10^8 \text{ cfu/mL})\) of S3; (iv) 3 mL of conidia suspension \((1 \times 10^6 \text{ spores/mL})\) of *F. oxysporum* and 3 mL of 45% prochloraz at a concentration of 450 µg/mL; (v) 5 mL suspension \((1 \times 10^8 \text{ cfu/mL})\) of S3.
It is a catalase-producing anaerobic bacterium that can grow on glucose, mannitol, fructose, sucrose, lactose, and cellobiose and not on arabinose, xylose, maltose, rhamnose, citrate, sorbitol, inositol, or malonate. Additionally, it hydrolyses casein, starch, and gelatine and not sulphur-containing amino acids. The nitrate reduction and V-P tests were positive, and the methyl red test was negative. The 16S rDNA sequence of Bv S3 deposited in GenBank (accession no. MW786667) was 100% identical to that of *B. velezensis* SB1216 (accession no. CP015417.1). The gyrB sequence of Bv S3 deposited in GenBank (accession no. OR990557) was 100% identical to that of *B. velezensis* PD9 (accession no. MT338276.1). Phylogenetic tree analysis showed that Bv S3 and *B. velezensis* have close genetic relationships (red color in Figure 4)(The strains and accession numbers of the phylogenetic tree are detailed in the Supplementary Material). Therefore, Bv S3 was identified as *B. velezensis* based on the morphological and molecular phylogenetic analyses.

**Figure 3.** Scanning electron micrograph of cells of *Bacillus velezensis* S3 grown at 30 °C in nutrient agar broth for 24 h.

**Figure 4.** Phylogenetic tree for *Bacillus velezensis* isolate S3 identification based on 16S rRNA gene and gyrB gene. The bootstrap values on the branching nodes were calculated on 1000 replications. Phylogenetic trees of Bv S3 were constructed based on the Bayesian inference (BI) method.

3.4. Stability of the Antifungal Substances in Bv S3

The antifungal substances in Bv S3 were insensitive to temperatures of 60 °C or lower and sensitive to temperatures above 80 °C. Still, they had certain antifungal activity (Figure 5A). The antifungal substances were sensitive to pH, with the strongest activity at pH 7.0 (Figure 5A). Longer UV treatment enhanced this antagonistic effect (Figure 5B).
Figure 5. Stability of antifungal substances of S3 at different temperatures, pH values, and ultraviolet treatment times. Sub-figures (A) means the treatment of different temperatures, pH values. Sub-figures (B) means the treatment of different ultraviolet treatment times. Error bars indicate standard errors of the means of two repeated experiments. Control was not exposed to UV. Different letters above the bars indicate significant difference within each treatment group (i.e., temperature, pH value, and ultraviolet treatment time) according to Duncan’s multiple range test ($p < 0.05$). The df values, F-value, and $p$-value from the ANOVA of the data in Figure 5 are displayed in the Supplementary Materials.

3.5. Effect of Bv S3 on the Hyphal and Conidia of F. oxysporum

The antagonistic active substances of Bv S3 at different doses significantly inhibited conidial germination and hyphal growth of F. oxysporum (Figure 6). At a concentration of 10%, Bv S3 inhibited spore germination by 66.7% and hyphal growth by 45.7%. The mycelium of F. oxysporum treated with Bv S3 filtrate showed protoplasmic aggregation, swelling deformation, and folding (Figure 7). Figure 7 shows the mycelium, healthy mycelia, and even distribution of mycelia cells were observed in the control group (Figure 7A); in the 1% treatment group, slight deformation of the mycelia and reduction of the diaphragm between the mycelia were observed (Figure 7B). When the concentration increased to 5%, the mycelial protoplasts gathered and the mycelial cells were significantly distorted (Figure 7C); in the 10% treatment group, the growth point of the mycelia was swollen and deformed, the cytoplasm overflowed, and the mycelia could not grow normally (Figure 7D). These effects became more noticeable with increasing concentrations of the Bv S3 filtrate.

3.6. Effects of Bv S3 Treatments on the Activities of Soil Neutral Phosphatase, Urease, Cellulase, Invertase, and Catalase

The Bv S3 suspension had no impact on S-CL activity (Figure 8A) and significantly improved the activities of S-NP (Figure 8B), S-CAT (Figure 8C), S-UE (Figure 8D), and S-SC (Figure 8E). F. oxysporum inoculation in the soil significantly decreased S-CAT, S-UE, and S-SC activities. Thus, the application of Bv S3 repaired the damage caused by F. oxysporum to soil activity.
Figure 6. Effect of Bv S3 filtrate on conidial germination and mycelial growth of *Fusarium oxysporum*. Bv S3 filtrate was applied at 1, 5, and 10%. Error bars indicate standard errors of the means of two repeated experiments. Different letters above the bars indicate a significant difference within each group (i.e., conidial germination and mycelial growth) according to Duncan’s multiple range test (*p* < 0.05). The df values, F-value, and *p*-value from the ANOVA of the data in Figure 6 are displayed in the Supplementary Materials.

Figure 7. Effect of Bv S3 filtrate on mycelial morphology of *Fusarium oxysporum* (*×*400 times). (A) non-treated control; (B–D) indicate treatments at different concentrations (1%, 5%, and 10%, respectively) for 14 h. The arrows represent morphological changes in mycelial cells following various treatments.

3.6. Effects of Bv S3 Treatments on the Activities of Soil Neutral Phosphatase, Urease, Cellulase, Invertase, and Catalase

The Bv S3 suspension had no impact on S-CL activity (Figure 8A) and significantly improved the activities of S-NP (Figure 8B), S-CAT (Figure 8C), S-UE (Figure 8D), and S-SC (Figure 8E). *F. oxysporum* inoculation in the soil significantly decreased S-CAT, S-UE, and S-SC activities. Thus, the application of Bv S3 repaired the damage caused by *F. oxysporum* to soil activity.
Figure 8. Effect of S3 on (A) soil cellulase (S-CL), (B) soil neutral phosphatase (S-NP), (C) soil catalase (S-CAT), (D) soil urease (S-UE), and (E) soil invertase (S-SC). Four treatments with 3 replicates were included: (i) 3 mL of sterile water as a control; (ii) 3 mL of conidia suspension (1 × 10⁶ spores/mL) of Fusarium oxysporum; (iii) both 3 mL of conidia suspension (1 × 10⁶ spores/mL) of F. oxysporum and 5 mL suspension (1 × 10⁸ cfu/mL) of Bv S3; (iv) 3 mL of conidia suspension (1 × 10⁶ spores/mL) of F. oxysporum and 3 mL of 45% prochloraz at a concentration of 100 µg/mL. Error bars indicate standard errors of the means of two repeated experiments. Different letters above the bars indicate significant differences in different treatments (i, ii, iii, iv) over the same period (p < 0.05). The df values, F-value, and p-value from the ANOVA of the data in Figure 8 are displayed in the Supplementary Materials.

4. Discussion

Numerous soil-borne plant pathogens have been controlled using Bacillus spp. as biological control agents [31]. The first case of B. velezensis infection was reported in Spain.
in 2005 [47]. According to Wang et al. [48,49], biochemical characterisation, 16S rRNA and gyrB sequencing, and phylogenetic analysis can be used to identify B. velezensis, consistent with the identification method used in this study. Based on these traits, the bacterial strain Bv S3 was identified as B. velezensis in this study.

The use of beneficial microbes to control plant diseases has advanced rapidly over the past 20 years [50]. Studies have discussed the application of biofactors in RSB regulation, including Trichoderma virens [51], Streptomyces platensis [52], Burkholderia heleia [17], bacteriophages [53], Phoma sp. [15], Trichoderma harzianum [15], and Pseudomonas sp. [54]. Compared with other soil-borne diseases in crops, there is still a lack of reports on the screening and application of biocontrol agents against RSB. B. velezensis is a crucial component of plant growth-promoting rhizobacteria that promotes plant development and prevents soil-borne illnesses [31,55]. In addition to controlling pathogenic fungi, microorganisms isolated from the soil rhizosphere promote plant development and health and systemic resistance [56,57]. According to Alsohim’s research, the advantages of inoculating soil isolates of bacteria into the rhizosphere of plants are that they are easily adapted and succession [58]. According to the findings of Agaras et al., Moto et al., and Chavez-Diaz et al., in contrast to plant endophytes, biocontrol bacteria isolated from soil have advantages in antagonizing pathogenic fungi in the soil, colonizing in the rhizosphere, and improving rice rhizosphere microecology [59–61].

Bacillus spp. are efficient biological control agents because they produce various antifungal lipopeptides. To identify microorganisms that are more beneficial for biocontrol, we screened bacterial strains, performed research on the development of biocontrol agents, and observed that B. velezensis (Bv. S3) exhibited good biocontrol potential against RSB. Bv S3 demonstrated a strong antagonistic effect against several pathogenic fungi, including F. incarnatum, F. redolens, F. equiseti, F. solani, F. tricinctum, Chaetomium globosum, F. proliferatum, and F. petroliphilum. These results indicated that Bv S3 is more valuable for the biological control of field crop diseases caused by multiple pathogens. To our knowledge, this is the first study to report the use of B. velezensis isolated from the rice rhizosphere for the biological control of RSB.

According to several studies, B. velezensis can reduce the severity of plant diseases, and its secondary metabolites can prevent pathogenic fungi from attacking plants [62,63]. Additionally, it has been extensively documented that Bacillus strains generate a family of lipopeptides known as surfactins, iturins, and fengycins [35,64]. Iturin family compounds have a broad antifungal range and low toxicity, making them ideal biological control agents for disease management in many crops [35,65,66]. Lipopeptides generated by B. velezensis were observed to have a stronger inhibitory effect against rice blast disease caused by Magnaporthe oryzae and to promote irregular mycelial morphology [55]. The filtrate of B. velezensis can inhibit fungal hyphal growth and spore germination. Additionally, it can distort hyphae and rupture the hyphal cell protoplasm in fungi [67–69]. Surfactin A, iturin A, and fengycin B are the primary antifungal metabolites produced by B. velezensis [66]. Surfactin A mostly inhibits fungal mycelial development and spore germination. In contrast, iturin A inhibits mycelial growth and causes exocytosis of fungal protoplasts, resulting in spore deformities. Iturin A and surfactin A can trigger defence genes in host plants, decreasing disease symptoms [70–73]. Fengycin B induces structural changes in fungal spores [74]. A wide diversity of antifungal metabolite enzymes have been commercialised, dramatically lowering environmental pollution [70,72].

These findings are consistent with the results of our study. In this study, the filtrate of Bv S3 dramatically decreased mycelial development and spore germination in F. oxysporum, thereby increasing spore deformation. Thus, we believe that the inhibitory components of B. velezensis S3 are mostly surfactin A and iturin A. Temperature, pH, and UV radiation were measured in this study because lipopeptides are more susceptible to these indices. Lipopeptides lose much of their antifungal activity at high temperatures and in highly acidic and alkaline environments. After being treated at a high temperature of 121 °C, the sterile filtrate of Bv S3 still had some antifungal activity, with an inhibition rate of 44.23%
against the fungus. These results are consistent with those of Wang et al. [75], Hwang et al. [76], and Huang et al. [77].

Continuous UV exposure increases the efficacy of antifungal agents. The structure of antifungal substances is altered following UV irradiation, leading to the amplification of antifungal activity, which demonstrates the dominance of Bv S3 in this regard; its mechanism of action will be investigated in future research. These findings were consistent with those of Li et al. [39], Li et al. [12], and Xu et al. [78].

_Bacillus velezensis_ promotes plant growth and prevents the spread of soil-borne diseases [31]. According to Robert et al., Mariane et al., and Gousterova et al., soil quality is typically connected to the activity of beneficial soil microbes, and measuring soil enzyme activity is frequently advised when evaluating soil quality. This indicator can be used as a value assessment for biological management [79–82]. _B. velezensis_ S3 considerably increases the activities of S-SC and S-UE, according to Lu et al. [83]. Wei et al. [84] reported that _B. velezensis_ increased S-UE, S-NP, and S-SC activities. Singh et al. [85] reported that adding three biocontrol strains, including _B. velezensis_, significantly increased S-CAT activity in the soil. In this study, we observed that Bv S3 bacterial suspension significantly improved the activities of S-NP, S-CAT, S-UE, and S-SC. This finding is consistent with those of the previous studies.

Next, the active antifungal substances of Bv S3 were analysed. The primary antifungal substances will be determined, their physical properties (such as stability in the natural environment) studied, and their effectiveness confirmed by mixing them with various fungicides and applying them to the soil. These results provide theoretical support for developing more efficient, less toxic, and more stable biological agents.

### 5. Conclusions

A biocontrol bacterial strain was identified and screened in rice seedling blight-affected fields. This bacterium demonstrated control effects against the dominant strain of rice-seedling blight in Heilongjiang Province, China, _F. oxysporum_. The bacterium was identified as _B. velezensi_ and named Bv S3. Bv S3 has a broad spectrum of inhibition against a wide range of common plant pathogenic fungi. In contrast, its sterile filtrate inhibits the conidial germination and mycelial growth of _F. oxysporum_. Bv S3 effectively reduced RSB caused by _F. oxysporum_, with an average efficacy of 71.0%, as shown through potting tests. In addition, the active antifungal substance produced by Bv S3 showed the best inhibitory effect at pH 7, and the antifungal activity of Bv S3 was increased by extending UV irradiation. Application of Bv S3 to the soil improved soil quality deterioration and increased the activity of neutral phosphatase, urease, invertase, and catalase in rice rhizosphere soil. Therefore, we comprehensively evaluated Bv S3 to control RSB, demonstrating its potential for application in controlling RSB during rice production.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/agronomy14010167/s1](https://www.mdpi.com/article/10.3390/agronomy14010167/s1).

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