horticulturae

MDPI

Article

Identification of Two *Bacillus* Strains with Antimicrobial Activity and Preliminary Evaluation of Their Biocontrol Efficiency

Erfeng Li [1,†], Yuxin Li [1,†], Xinyu Dai [1], Wanrong Yan [2,*] and Gang Wang [3,*]

[1] Horticulture and Landscape College, Tianjin Agricultural University, Tianjin 300392, China
[2] Institute of Plant Protection, Hainan Academy of Agricultural Sciences (Research Center of Quality Safety and Standards for Agricultural Products of Hainan Academy of Agricultural Sciences), Haikou 571100, China
[3] Key Laboratory of Agro-Products Quality and Safety Control in Storage and Transport Process, Ministry of Agriculture and Rural Affairs, Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China
* Correspondence: yanwanrong818@163.com (W.Y.); wanggang02@caas.cn (G.W.); Tel.: +86-183-8928-6277 (W.Y.); +86-155-0115-0911 (G.W.)
† These authors contributed equally to this work.

Abstract: Cabbage Fusarium Wilt (CFW) is a serious disease caused by *Fusarium oxysporum* f. sp. *conglutinans* in many parts of the world. The use of chemical fungicides has placed a heavy burden on the environment and is prone to drug resistance in plant pathogens. As a method with great potential, biological control has attracted the attention of many academics both at home and abroad. In this study, we have found that strains B5 and B6 had a strong inhibitory effect on various pathogens and significantly inhibited mycelium growth. They were both identified as *Bacillus velezensis* by morphological features, biochemical determinations, 16S rRNA gene and *gyrA* gene sequence analysis. When different concentrations of bacterial suspension were applied to cabbage seeds, hypocotyl and taproot length increased to varying degrees. The in vivo results showed that B5 and B6 decreased the incidence of cabbage seedling wilt disease, with B6 performing significantly better. Furthermore, *B. velezensis* B6 had the ability to colonize cabbage plants and rhizosphere soil. Thus, strain B6 has great potential for biocontrol development and this research could lead to the development of a promising biological agent for CFW.

Keywords: *Bacillus velezensis*; antimicrobial activity; growth-promoting effect; biocontrol efficiency

Citation: Li, E.; Li, Y.; Dai, X.; Yan, W.; Wang, G. Identification of Two *Bacillus* Strains with Antimicrobial Activity and Preliminary Evaluation of Their Biocontrol Efficiency. *Horticulturae* **2022**, *8*, 744. https://doi.org/10.3390/horticulturae8080744

Academic Editors: Vladimir K. Chebotar and Anastasiya M. Kamionskaya

Received: 20 July 2022
Accepted: 15 August 2022
Published: 18 August 2022

1. Introduction

Fusarium wilt is a general term for a class of plant diseases caused by pathogenic fungi that wilt and kill plants. One of the most common fungal pathogens is *Fusarium oxysporum*. Cabbage Fusarium wilt (CFW) is a soil-borne disease caused by *Fusarium oxysporum* f. sp. *conglutinans*, which was first discovered in the United States in 1895 [1]. In the next few decades, CFW was reported in Japan, China, India and other countries around the world. In China, CFW was first found in Yanqing County, Beijing [2]. Since then, the damaged area has been expanding, threatening about one third of the summer and autumn cabbage planting areas in northern China, which has become a major resistance to the production of cruciferous vegetables [3]. At present, the prevention and control of CFW is still dominated by chemical fungicides, but this increases the risk of fungal drug resistance, and has high residual toxicity, which adversely affects both the environment and human health. The main method of disease control in agriculture is the breeding of disease-resistant varieties. This method, however, is slow to take effect and is susceptible to seasonal or geographical constraints. Pathogenic microorganisms may also cause variations and other problems, affecting the ability of plants to control pathogens. As a result, biological control, such as using plant extract and endophytic bacteria against pathogens [4], has received increased

attention as a safe and environmentally friendly control method, and the research on antagonistic microorganisms has also entered a new stage [5,6].

In recent years, the microorganisms used to control Fusarium wilt disease mainly include *Bacillus* [7], *Penicillium* [8], *Pseudomonas* [9] and *Streptomyces* [10], etc., among which *Bacillus* has advantageous characteristics such as heat tolerance, salt tolerance and UV resistance [11–13]. Several *Bacillus* spp. have been developed as commercial bio-pesticides because of their multi-layer cell wall structure and ability to produce endophytic spores and long-term survival after treatment in the natural environment [14,15]. They are widely used in agricultural production and are of great significance in the biological control of plant diseases. *Bacillus* spp. are capable of producing two classes of antagonistic factors, lipopeptide antibiotics and antagonist proteins, which have antibiotic effects against plant pathogens [16]. Lipopeptides belong to a class of microbial polypeptides that activate the defense mechanisms of plants [17]. Lipopeptide antibiotics synthesized by the non-ribosomal pathway can be classified into surfactins, iturins and fengycins, which have a good inhibitory effect on bacteria, fungi and viruses [18]. Surfactin can induce resistance in plants, the capacity of *Bacillus subtilis* S499 to generate surfactin is positively related to its resistance to *Botrytis cinerea* [19]. Iturin plays an important role in the biological control of plant pathogens due to its hemolytic, surface active and potent antifungal properties. It is an important antimicrobial active substance that causes cytolysis and cytoplasmic efflux of *Verticillium dahliae* [20]. *B. subtilis* UTBSP1 has the ability to produce fengycin, which can inhibit the growth of *Aspergillus flavus* [21]. Antagonistic protein-based antimicrobial substances of *Bacillus* mainly include cell-wall-degrading enzymes such as chitinases and glucanases, as well as bacteriocins [22]. Fungal cell walls were broken down by cell-wall-degrading enzymes released by *B. atrophaeus* JZB120050, such as chitinase and glucanase [23]. Pei et al. found that the bacteriocin RC20975 was able to cause extensive cell damage and bacterial lysis against *Alicyclobacillus acidoterrestris* [24]. Bacteriocins were also found to inhibit plant pathogenic bacteria and nematodes in recent studies [25,26]. Therefore, *Bacillus* is a biocontrol bacterium with good application prospects.

In this study, we identified two *B. velezensis* strains B5 and B6, detected their antagonistic activity on various plant pathogenic fungi and researched their effects on disease control against CFW and growth promotion in cabbage.

2. Materials and Methods

2.1. Microorganisms and Growth Condition

The biocontrol strains, used in this study, were isolated from healthy bitter melons (*Momordica charantia* L.) and deposited in the Plant Disease Laboratory of Tianjin Agricultural University under accession number PATAU191011 and PATAU191012. The pathogens used in this study were provided by the Plant Pathology Laboratory of Tianjin Agricultural University. The fungi cultured in potato glucose agar (PDA) plates at 28 °C and bacteria cultured in LB (Luria-Bertani) plates at 37 °C.

2.2. Determination of Antifungal Activity

The pathogeny fungal plugs with a diameter of 5 mm were inoculated on the fresh PDA plates, cultured at 28 °C for 2 days. *Bacillus* suspension of 2 µL (1×10^8 CFU/mL) and pathogens were inoculated at the same distance on both sides as we reported [27]. As a control, PDA was only inoculated with the pathogen. Then, incubated at 28 °C for 3 days to observe and record the inhibition bandwidth. Each treatment was replicated three times.

2.3. Growth Condition of Pathogenic Fungi on Culture Medium Containing Different Concentrations of Bacillus

Strains B5 and B6 were inoculated on LB plates and incubated at 37 °C for 24 h. Single colonies were picked and inoculated into 100 mL of LB liquid medium and incubated for 20 h (37 °C, 180 rpm). Equivalent volume of the suspensions with specific concentration were transferred to a 50 mL centrifuge tube, which was filled with potato glucose agar

(PDA) medium to 20 mL and then mixed well. After that, it was, respectively, put into the Petri dishes to create the medium with the corresponding concentrations of 10^4, 10^5, 10^6, 10^7 and 10^8. Finally, we inoculated the pathogen plugs with 5 mm diameter, including *Fusarium oxysporum* f. sp. *conglutinans*, *Botrytis cinerea*, *Alternaria solani*, *F. oxysporum* f. sp. *zingiberi* and *Alternaria alternata*, in the center of the plates, respectively, and incubated at 28 °C. As a control, PDA was only inoculated with the pathogen. Each treatment was replicated three times. The colony diameters were measured after 7 days using the crossover method, and the inhibition rates were calculated for each treatment. The growth inhibition was calculated using the formula [28]:

$$\text{Percentage of growth inhibition} = (C - T)/C \times 100\%, \quad (1)$$

where C is the diameter of fungal colony in control and T is the diameter of fungal colony in the treatment.

2.4. Identification of B5 and B6

B5 and B6 strains were cultured in LB plates at 37 °C for 2 days. The morphological identification was determined by the colony characteristics. Physiological and biochemical identification was based on the manual of systematic identification of general bacteria [29]. The molecular identification was conducted as follows. The DNA was extracted with the bacterial DNA extraction kit. The 16S rRNA and *gyrA* genes were amplified to identify strain B5 and B6 [30,31]. The sequencing was completed by Shanghai Sangon Biotech Co., Ltd., China. The DNA sequence was analyzed by comparing it with those in NCBI GenBank using the BLASTN program based on the method of Altschul et al. [32]. The DNA multiple sequence homology analysis was performed using MEGA X software to construct a phylogenetic tree using the maximum likelihood method [33].

2.5. The Growth Promotion Ability of B5 and B6 in Cabbage Seedlings

The bacterial cultures were centrifugated at 5000 rpm for 15 min, and the precipitates were diluted by sterile water to obtain suspensions with concentrations of 10^6, 10^9 and 10^{12} CFU/mL. Cabbage seeds were soaked in above three concentrations of suspension for 40 min, sterile water was used as control, then placed in Petri dishes lined with wetted filter paper. Twenty seeds were in each treatment and incubated in a light/dark cycle at 25 °C. During the period, watering 1–2 times a day to keep humidity and provided a suitable culture environment for the seeds. After 4 days, hypocotyl length and taproot length were measured. The seed germination rate was calculated using the formula [34]:

$$\text{Germination rate} = n/N \times 100\%, \quad (2)$$

where n is the number of seed germinated and N is the total number of seeds planted.

2.6. Biocontrol Efficacy of B5 and B6 in Potted Cabbage Seedlings Inoculated with F. oxysporum

F. oxysporum was prepared as a suspension of 1×10^7 CFU/mL [7] and the bacterial suspension was prepared as 1×10^8 CFU/mL. Cabbage seedlings with 2–3 true leaves were inoculated by the root-dipping method [35]. Treatment A was the healthy cabbage plants inoculated with sterilized distilled water and these were used as the control group. There was other five treatments in this research. Treatment B was the cabbage plants inoculated with *F. oxysporum* only; Treatment C was the cabbage seedlings inoculated with *F. oxysporum* and watered with 30 mL B5 suspension in the concentration of 1×10^8 CFU/mL into soil; Treatment D was the cabbage seedlings inoculated with *F. oxysporum* and watered with 30 mL B6 suspension in the concentration of 1×10^8 CFU/mL into soil; Treatment E was the cabbage seedlings inoculated with the suspension, including *F. oxysporum* and B5; and treatment F was the cabbage seedlings inoculated with the suspension, including *F. oxysporum* and B6. In total, 70 g of soil was weighed and the seedlings were replanted in the seedling pots and maintained in a growth chamber under the condition of 28 °C

for 16 h light and 8 h dark. Ten days after inoculation, the disease was recorded and the disease index was calculated [7]. Disease symptoms were assessed based on the disease rating scales [35]. The disease index (DI) and control efficacy were calculated using the formulas [36]:

$$DI = (a \times 0 + b \times 1 + c \times 2 + d \times 3 + e \times 4 + f \times 5)/[(a + b + c + d + e + f) \times 5] \times 100, \quad (3)$$

where a, b, c, d, e and f are the number of cabbage plants in the same disease rating scale.

$$\text{Control efficacy (\%)} = (C - T)/C \times 100\%, \quad (4)$$

where C refers to the disease index of the control group and T denotes the disease index of the treatment group.

2.7. Colonization Characteristics of B6 in Cabbage Seedlings and Soil

To avoid the growth of various bacteria in the cabbage seedlings and soil, the natural antibiotic labeling method was used as described previously [37]. The B6 suspension was spread on LB medium with 2 μg/mL of kanamycin and incubated at 37 °C for 24 h. The colonies with the same morphology as the original strain were selected and transferred to LB medium with 5 μg/mL of kanamycin, then incubated for 24 h. This was repeated to prepare the B6 strain which could grow on LB medium with 10, 15, 20, 25, 50, 75 and 100 μg/mL of kanamycin. The cabbage seedlings were treated by root irrigation with 10 mL of B6 suspension in the concentration of 3×10^8 CFU/mL. The roots, stems and leaves of the cabbage seedlings were, respectively, sampled 1, 4, 7, 11 and 15 days after root irrigation. The six plants were taken as a group, and 2 g of the roots, stems and leaves of each plant was taken, which were ground into a homogenate after washing and diluted to 1×10^2, 1×10^3, 1×10^4 and 1×10^5 times. The 100 μL supernatant was taken, respectively, and spread evenly on antibiotic LB plates and incubated for 48 h to observe the number of colonies. Similarly, the rhizosphere soil of the cabbage seedlings was taken on the 1st, 4th, 7th, 11th and 15th day and placed in a triangular flask containing 10 mL of 0.85% NaCl solution with shaking culture at 160 rpm for 30 min. The supernatant was diluted 1×10^2, 1×10^3, 1×10^4 and 1×10^5 times, respectively, and 100 μL was taken and spread evenly on antibiotic LB plates. The colony number was observed after 48 h incubation.

2.8. Statistical Analysis

The experimental data were subjected to variance analysis (ANOVA) using the SPSS software version 19.0 (SPSS Inc., Armonk, NY, USA). Different treatment mean values were separated using Duncan's multiple range test at $p < 0.05$.

3. Results

3.1. Determining the Antimicrobial Spectrum of Strains B5 and B6

The inhibitory effects of the strains B5 and B6 on the seven pathogenic fungi were shown in Table 1. B5 and B6 had different antagonistic abilities against the pathogens, but they all showed good inhibition effects. Strains B5 and B6 had a significant antagonistic effect on Fusarium wilt disease on watermelon, cotton and cucumber, and demonstrated fungal inhibitory bandwidths more than 10 mm. Apart from that, B5 also exhibited obvious inhibitory effect against *F. oxysporum* f. sp. *conglutinans*, *Botrytis cinerea* and *Alternaria alternata*, while B6 had an obvious inhibitory effect on *F. oxysporum* f. sp. *conglutinans.* Therefore, B5 and B6 both have great potential for biocontrol development on plant Fusarium wilt.

3.2. Inhibition Effect of B5 and B6 Strains at Different Concentrations on Plant Pathogens

The strains B5 and B6 exhibited antagonistic activities against the five-tested fungal pathogens on PDA plates. In general, the diameter of pathogen colonies was negatively correlated with the concentration of bacterial suspension contained in the plates, namely, the higher the concentration, the smaller the diameter of the pathogen colonies compared

to the control group (Figure 1). This indicated that B5 and B6 both had significant inhibitory effects on each pathogen.

Table 1. Antifungal activity of B5 and B6 against different pathogens in vitro.

Strains	Inhibition Effects [1]						
	F. oxysporum* f. sp. *niveum	***F. oxysporum* f. sp. *vasinfectum***	***F. oxysporum* f. sp. *cucumerinum***	***F. oxysporum* f. sp. *conglutinans* Race 1**	***F. oxysporum* f. sp. *conglutinans* Race 2**	***Botrytis cinerea***	***Altenaria alternata***
B5	+++	+++	+++	++	++	++	++
B6	+++	+++	+++	++	++	+	+

[1] Mean values of inhibitory zone diameters were obtained from three replicates for each treatment. The inhibitory zone diameters of <5 mm, 5−10 mm and >10 mm were shown as +, ++ and +++, respectively.

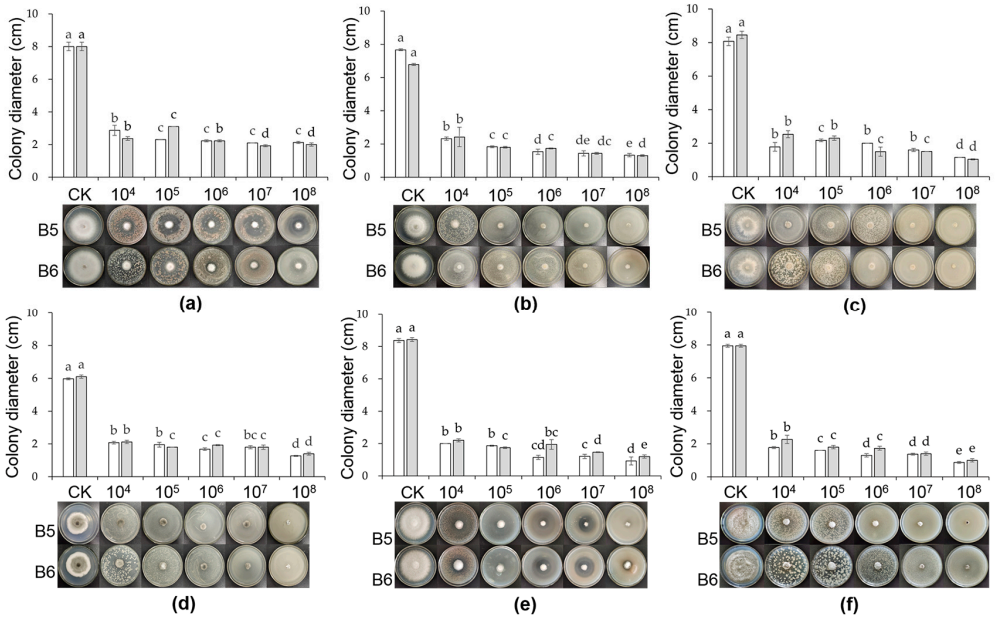

Figure 1. Inhibition effects of strain B5 (white rectangle) and B6 (grey rectangle) against *F. oxysporum* f. sp. *conglutinans* race 1 (**a**), *F. oxysporum* f. sp. *conglutinans* race 2 (**b**), *Botrytis cinerea* (**c**), *Alternaria solani* (**d**), *F. oxysporum* f. sp. *zingiberi* (**e**) and *A. alternata* (**f**). Values were means of three replicates. Error bars represented standard error of the mean. For each pathogen, different lowercases above the columns indicated statistical difference at $p < 0.05$.

With the increasing concentration of B5 and B6 suspension in the cultural medium, the inhibition rate was generally on the rise. *F. oxysporum* f. sp. *conglutinans* race 1 reached the maximum inhibition rate of 73.75% at the concentration of 10^7, but there was no significant difference in the inhibition rate with 10^8. The rest of the pathogens were inhibited most significantly at 10^8, and the inhibition rates were all above 75%, indicating that the higher the concentration, the greater the inhibition effect.

3.3. Identification of B5 and B6

The colonies of strains B5 and B6 on LB plates both showed a creamy yellow color, with an opaque, moist and smooth surface and untidy edges (Figure 2a). The single colony is nearly circular (Figure 2b). They are Gram-positive bacteria and capable of producing spores. They can grow well in a temperature of 28–37 °C. They were positive for methylred staining, the Voges-Proskauer test, hydrolysis of starch, citrate utilization and sucrose fermentation. They also had NaCl resistance, with growing occuring with 0–8% NaCl. Furthermore, they were negative for carboxymethyl cellulose and phenylalanine deaminase production.

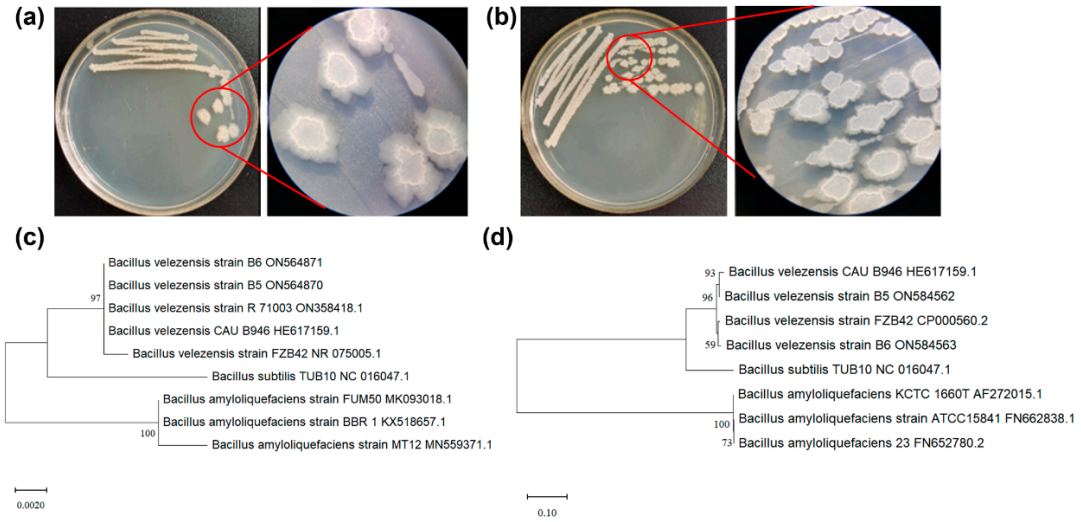

Figure 2. The morphological and molecular identification of strains B5 and B6. Morphological and microscopic observation of strains B5 (**a**) and B6 (**b**). Phylogenetic tree based on the sequences of 16S rRNA gene (**c**) and *gyrA* gene (**d**).

The strains B5 and B6 were tested with genomic DNA as a template, and the PCR products were detected by agarose gel electrophoresis. The 16S ribosomal RNA gene and *gyrA* gene fragments were obtained by sequencing, respectively, which were homology matched by BLASTN in NCBI, and phylogenetic evolutionary trees were constructed. The phylogenetic trees demonstrated that B5 and B6 strains formed a cluster closely related to *B. velezensis* pattern strains CAU B946 and FZB42, indicating that B5 and B6 strains belong to the species *B. velezensis* (Figure 2c,d). Combining the morphological characteristics and molecular identification results, B5 and B6 were identified as *B. velezensis*. The 16S ribosomal RNA gene sequence and partial *gyrA* of B5 and B6 were deposited in the GenBank database with the accession numbers ON564870, ON564871, ON584562 and ON584563, respectively.

3.4. Growth Promoting Ability of B5 and B6 in Cabbage Seedlings

To investigate the effect of B5 and B6 on seed germination and growth, cabbage seeds were treated with different concentrations of B5 and B6 suspension, sterile water was used as a control. They significantly improved the germination rate and seed viability to different degrees (Table 2). The hypocotyl length and taproot length under the treatment of B6 suspension with 10^{12} CFU/mL were the longest, with 1.25 cm and 2.55 cm, respectively (Figure 3). The seed germination rate increased the concentration of suspension increased. The germination rate of B5 or B6 suspension in concentrations 10^{9} and 10^{12} CFU/mL was 100%. To summarize, B5 and B6 suspension could promote cabbage germination and growth significantly, and B6 performed better.

Table 2. Effect of B5 and B6 suspension at different concentrations on cabbage seed germination.

	Treatment (CFU/mL)						
	CK	**B5**			**B6**		
		10^{6}	**10^{9}**	**10^{12}**	**10^{6}**	**10^{9}**	**10^{12}**
Germination rate	85% a	90% a	100% b	100% b	90% a	100% b	100% b

Different lowercases indicated statistical difference at $p < 0.05$.

3.5. Biocontrol Efficacy of B5 and B6 in Cabbage Seedlings Inoculated with F. oxysporum

The disease assessment was conducted 10 days after inoculation. The disease index of each treatment group was calculated separately according to the disease rating scales.

There was no disease symptoms for treatment A (Figure 4a). Compared with treatment B, the disease index of Fusarium wilt in cabbage was significantly reduced under the effects of B5 and B6 suspension (Figure 4). There was no significant difference in disease indexes and control effects among treatments C, D and E. The control effects were all less than 35%. Treatment F, the roots dipped with B6 suspension, had a good control effect that reached 55.18%. The disease index also decreased significantly, which was 27.08 (Table 3). Therefore, we chose B6 for further research on its colonization characteristics in cabbage and soil.

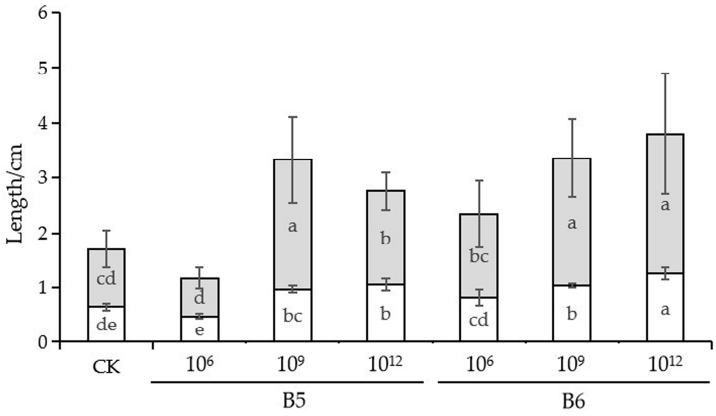

Figure 3. The hypocotyl length (white rectangle) and taproot length (grey rectangle) under different treatments. Different lowercases indicated statistical difference at $p < 0.05$.

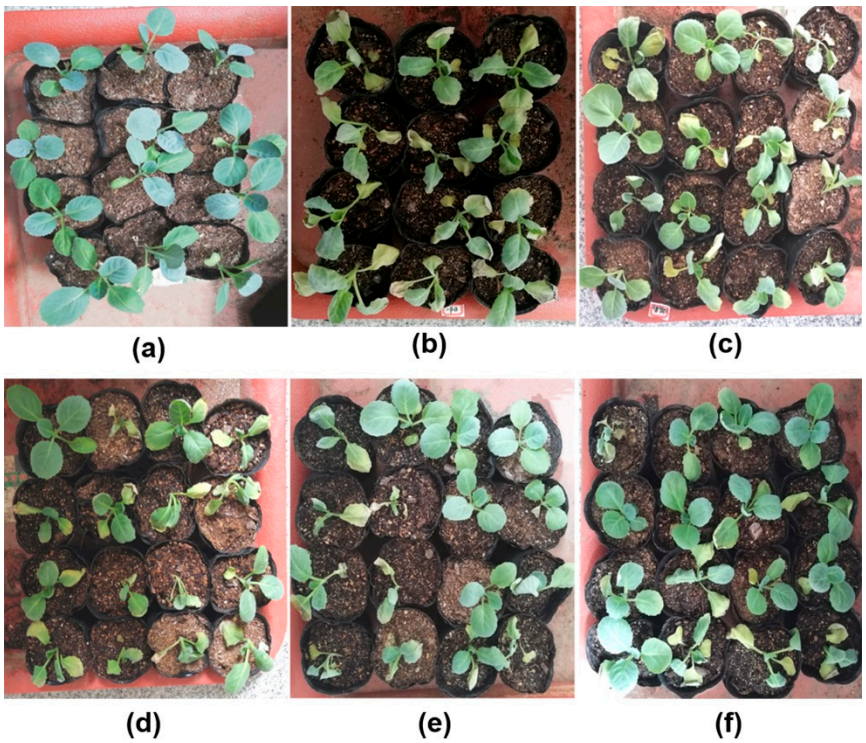

Figure 4. The disease symptoms of cabbage seedlings under different treatments. (**a**) Treatment A, which refers to cabbage seedlings inoculated with sterilized distilled water as a control group; (**b**) Treatment B, which refers to cabbage seedlings inoculated with *F. oxysporum* only; (**c**) Treatment C, which refers to cabbage seedlings inoculated with *F. oxysporum* and watered with B5 suspension into soil; (**d**) Treatment D, which refers to cabbage seedlings inoculated with *F. oxysporum* and watered with B6 suspension into soil; (**e**) Treatment E, which refers to cabbage seedlings inoculated with the suspension, including *F. oxysporum* and B5; (**f**) Treatment F, which refers to cabbage seedlings inoculated with the suspension, including *F. oxysporum* and B6.

Table 3. Control effects of B5 and B6 on cabbage Fusarium wilt.

Treatments [1]	Disease Index	Control Effect/%
A	0	–
B	60.42 a	–
C	39.58 b	34.49 a
D	47.92 b	20.69 a
E	43.75 b	27.59 a
F	27.08 c	55.18 b

[1] Treatment A refers to plants inoculated with sterilized distilled water as a control. Treatment B refers to plants inoculated with *F. oxysporum* only. Treatment C/D refers to plants inoculated with *F. oxysporum* and watered with B5/B6 suspension into soil. Treatment E/F refers to plants inoculated with the suspension, including *F. oxysporum* and B5/B6. Different lowercases indicated statistical difference at $p < 0.05$.

3.6. Colonization Characteristics of B6 in Cabbage Seedlings and Soil

The colonization of biocontrol bacteria is essential for its biocontrol efficacy. Figure 5 showed the colonization of B6 in cabbage seedlings and soil. It could colonize in roots, stems and leaves, especially in roots. In total, 7 days after irrigation, the number of colonies isolated from the roots, stems and leaves reached the maximum, which was 12×10^5 CFU/g, 1.9×10^5 CFU/g and 0.5×10^5 CFU/g, respectively. After that, colonies started to gradually decline and disappeared on the 15th day. In addition, the number of colonies isolated from the seedlings was "root > stem > leaf" in the days after irrigation. Overall, 3×10^5 CFU/g was monitored in the roots as early as the first day after root irrigation. At this time, B6 had not been detected in the stems and leaves. Figure 5d showed that B6 also colonized in rhizosphere soil. In general, the colonization amount of B6 in rhizosphere soil was the highest for the whole experiment. The number of colonies decreased gradually with time after root irrigation, with the greatest decrease of 25% on the 7th day until it decreased to 0 after 15 days. As a result, B6 could colonize effectively in the cabbage host and rhizosphere soil for more than 11 days.

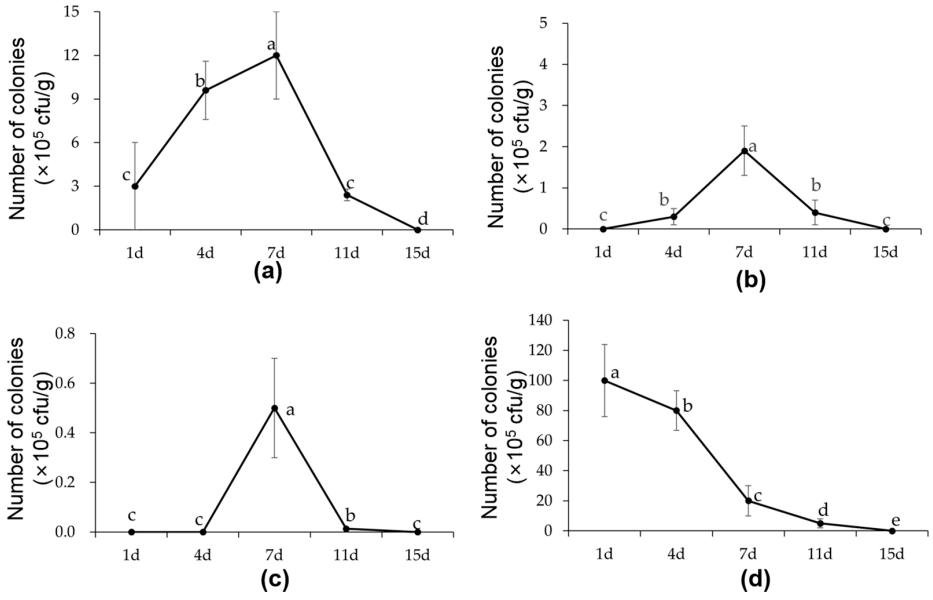

Figure 5. Colonization of B6 in the roots (**a**), stems (**b**), leaves (**c**) and rhizosphere soil (**d**) of cabbage seedlings. Different lowercases indicated statistical difference at $p < 0.05$.

4. Discussion

In recent years, biological control technology has become an important initiative for plant disease control in modern agricultural production. Due to the ability of *Bacillus* to secrete a variety of antifungal substances, it has become one of the most popular strains in the field of biocontrol. Studies showed that under the artificial control condition, the

application of biocontrol bacteria can often achieve good control effects [38]. It has been an effective strategy to screen biocontrol strains from plants. We have isolated microbes from various plants, among which *B. velezensis* strains B5 and B6 demonstrated excellent effects. In this study, the two strains had high antifungal activities, which could act against a variety of pathogens on different hosts, not only on cabbage. These findings suggested that they were potential biocontrol agents in agriculture.

Furthermore, B5 and B6 both had significant roles in promoting growth. The germination rate of seeds treated with B5 and B6 in concentrations over 10^9 CFU/mL was 100%. For cabbage roots, the growth effect become better along with the increase in B6 concentration, while the situation of B5 is different. The results showed that 10^9 CFU/mL of B5 was the most suitable for the growth of cabbage seeds, and there was no significant difference between 10^9 CFU/mL and 10^{12} CFU/mL from the germination rate. Liu et al. (2020) found that *B. methylotrophicus* DD-1 may create a range of plant growth inducers, including Indole-3-acetic acid (IAA), Gibberellic acid (GA) and Siderophore, which can improve the dry weight, root length and shoot length of rice seedlings [39]. This study showed the increase in the bacterial concentration promoted the growth of plants. That may be due to the production of antifungal substances or growth regulators. Microbial mechanisms for promoting plant growth in practical application including nitrogen fixation [40], phosphorus solubilization [41] and secretion of plant hormones [39], etc. Additionally, several systems frequently cooperate. *B. cereus* YN917 was reported to promote seed germination and seedling plant growth by producing siderophores, ACC deaminases, indole-3-acetic acid (IAA), phosphate solubilizing and enzymes such as β-1,3-glucanase, protease, cellulase and amylase [42]. Overall, the mechanism is complex and diverse, which should be explored further.

In addition, our results also showed that *B. velezensis* B5 and B6 decreased the incidence of cabbage seedlings' wilt disease. When the roots were dipped with B6 suspension, the control efficacy reached 55.18%. The disease index also decreased significantly, which was 27.08. Several previously described potential biocontrol agents have similar or higher efficacy. For example, the biocontrol efficacy of *Paenibacillus polymyxa* HX-140 against Fusarium wilt of cucumber seedlings was reported to be 55.6 % in 2021 [43].

B. velezensis B6 was able to colonize cabbage seedlings efficiently and rapidly. During this process, bacteria can exploit a number of strategies to enhance colonization, such as surfactin production [44], cell-wall-degrading enzyme production [45] and biofilm formation [46,47]. The production of multiple lipopeptides enhances and broadens the antifungal and antibacterial spectrum of *Bacillus* and facilitates the colonization of ecological niches [48]. In this study, the maximum amount of colonization in the root, stem and leaf was 12×10^5 CFU/g, 1.9×10^5 CFU/g and 0.5×10^5 CFU/g, respectively, and even up to 100×10^5 CFU/g in the rhizosphere soil. This suggested that the B6 strain could mainly colonize in the root and rhizosphere soil during the process of colonization, which was consistent with the conclusions of Coy's study [49]. Biocontrol bacteria could compete with pathogens for sites and play roles in disease control [50]. The amount of colonization in the rhizosphere soil showed a continuous decreasing trend, but increased in the roots, stems and leaves, indicating that B6 may transferred from the rhizosphere soil. Then, after 7 days, the colonies isolated from the roots, stems and leaves started to gradually decline and disappeared on the 15th day. This phenomenon indicated that the activity of the strain B6 was decreasing after 7 days, which led to weaker effects on cabbage seedlings. Since *F. oxysporum* is often infected from the root [51] and B6 could colonize the root, root irrigation treatment during cabbage planting could form an effective biological barrier to prevent the invasion of *F. oxysporum*. In this study, *B. velezensis* B6 was also found in the stems and leaves of cabbage, which proved that the B6 strain could be transferred from the roots to the stems and leaves, thus preventing the infection of pathogens more effectively. The colonization of *Bacillus* FZB42 on *Zea mays*, *Arabidopsis thaliana* and *Lemna minor* also showed similar results [52]. This strain was previously designated to *B. amyloliquefaciens* and reclassified to *B. velezensis* in 2018 [53]. The same was true for *B. velezensis* CAU B946,

which was previously known as *B. amyloliquefaciens* subsp. *plantarum* [53,54]. Another study found that *B. subtilis* EA-CB0575 not only effectively colonized banana and tomato rhizosphere, but also showed a certain role in promoting growth [55]. Endophytes colonize host plants and form a tight mutualistic symbiotic connection. It is critical to investigate the colonization and growth-promoting mechanisms of endophytes, as well as the molecular recognition mechanism between endophytes and plants, in order to fully exploit the entire function of the endophyte-plant microecosystem and ensure the long-term development of green agriculture.

In conclusion, we reported the biological control effects of two *B. velezensis* strains on CFW for the first time. This study has laid a theoretical foundation for the development and further application of *B. velezensis*; however, the above experiments were conducted in an indoor environment and the antagonistic bio-protection effect under natural conditions is easily affected by many factors such as soil, indigenous microorganisms and climate, etc. As a result, numerous experiments must be carried out to determine whether it can be successfully used as a biocontrol bacterium in actual production.

Author Contributions: Conceptualization, G.W., E.L. and W.Y.; methodology, E.L., Y.L. and X.D.; validation, E.L., Y.L. and X.D.; formal analysis, E.L. and Y.L.; data curation, E.L. and Y.L.; writing—original draft preparation, E.L. and Y.L.; writing—review and editing, G.W. and W.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Natural Sciences Foundation of China (grant number "31801682"), Scientific Research Project of Tianjin Municipal Education Commission (grant number 2017KJ179) and National College Students' Innovation and Entrepreneurship Training Program (grant number "202210061012").

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data sets supporting the results of this article will be freely available upon request to the first author.

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

References

1. Smith, E.F. The fungus infestation of agricultural soils in the United States. *Sci. Am. Suppl.* **1899**, *48*, 1981–1982.
2. Li, M.Y.; Zhang, T.T.; Li, X.H.; Yan, H. Wilt disease of cruciferous vegetables and its pathogen identification. *Plant Prot.* **2003**, *29*, 44–45. (In Chinese)
3. Liu, X.; Ling, J.; Xiao, Z.L.; Xie, B.Y.; Fang, Z.Y.; Yang, L.M.; Zhang, Y.Y.; Lv, H.H.; Yang, Y.H. Characterization of emerging populations of *Fusarium oxysporum* f. sp. *conglutinans* causing cabbage wilt in China. *J. Phytopathol.* **2017**, *165*, 813–821.
4. Gava, C.A.T.; Pinto, J.M. Biocontrol of melon wilt caused by *Fusarium oxysporum* Schlect f. sp. *melonis* using seed treatment with *Trichoderma* spp. and liquid compost. *Biol. Control.* **2016**, *97*, 13–20.
5. Phong, N.H.; Pongnak, W.; Soytong, K. Antifungal activities of *Chaetomium* spp. against Fusarium wilt of tea. *Plant Protect. Sci.* **2016**, *52*, 10–17. [CrossRef]
6. Fernando, W.G.D.; Ramarathnam, R.; Krishnamoorthy, A.S.; Savchuk, S.C. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol. Biochem.* **2005**, *37*, 955–964. [CrossRef]
7. Zhang, J.X.; Gu, Y.B.; Chi, F.M.; Ji, Z.R.; Wu, J.Y.; Dong, Q.L.; Zhou, Z.S. *Bacillus amyloliquefaciens* GB1 can effectively control apple valsa canker. *Biol. Control* **2015**, *88*, 1–7. [CrossRef]
8. Alam, S.S.; Sakamoto, K.; Inubushi, K. Biocontrol efficiency of Fusarium wilt diseases by a root-colonizing fungus *Penicillium* sp. *Soil Sci. Plant Nutr.* **2011**, *57*, 204–212. [CrossRef]
9. Rania, A.B.A.; Jabnoun-Khiareddine, H.; Nefzi, A.; Mokni-Tlili, S.; Daami-Remadi, M. Endophytic bacteria from Datura metel for plant growth promotion and bioprotection against Fusarium wilt in tomato. *Biocontrol Sci. Technol.* **2016**, *26*, 1139–1165. [CrossRef]
10. Wang, J.; Cai, B.Y.; Li, K.; Zhao, Y.K.; Li, C.Y.; Liu, S.W.; Xiang, D.D.; Zhang, L.; Xie, J.H.; Wang, W. Biological control of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in banana plantlets using newly isolated *Streptomyces* sp. WHL7 from marine soft coral. *Plant Dis.* **2022**, *106*, 254–259.
11. Obagwu, J.; Korsten, L. Integrated control of citrus green and blue molds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water. *Postharvest Biol. Technol.* **2003**, *28*, 187–194. [CrossRef]
12. Li, H.Q.; Yue, H.W.; Li, L.; Liu, Y.; Zhang, H.Y.; Wang, J.H.; Jiang, X.W. Seed biostimulant *Bacillus* sp. MGW9 improves the salt tolerance of maize during seed germination. *AMB Expr.* **2021**, *11*, 74. [CrossRef] [PubMed]

13. Nicholson, W.L.; Galeano, B. UV Resistance of *Bacillus anthracis* spores revisited: Validation of *Bacillus subtilis* spores as UV surrogates for spores of *B. anthracis* sterne. *Appl. Environ. Microb.* **2003**, *69*, 1327–1330. [CrossRef] [PubMed]
14. Hu, X.J.; Roberts, D.P.; Maul, J.E.; Emche, S.E.; Liao, X.; Guo, X.L.; Liu, Y.Y.; McKenna, L.F.; Buyer, J.S.; Liu, S.Y. Formulations of the endophytic bacterium *Bacillus subtilis* Tu-100 suppress *Sclerotinia sclerotiorum* on oilseed rape and improve plant vigor in field trials conducted at separate locations. *Can. J. Microbiol.* **2011**, *57*, 539–546. [CrossRef]
15. Santoyo, G.; Orozco-Mosqueda, M.C.; Govindappa, M. Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: A review. *Biocontrol Sci. Technol.* **2012**, *22*, 855–872. [CrossRef]
16. Yamamoto, S.; Shiraishi, S.; Suzuki, S. Are cyclic lipopeptides produced by *Bacillus amyloliquefaciens* S13-3 responsible for the plant defence response in strawberry against *Colletotrichum gloeosporioides*? *Lett. Appl. Microbiol.* **2015**, *60*, 379–386. [CrossRef]
17. Shafi, J.; Tian, H.; Ji, M.S. *Bacillus* species as versatile weapons for plant pathogens: A review. *Biotechnol. Biotechnol. Equip.* **2017**, *31*, 446–459. [CrossRef]
18. Schofield, B.J.; Skarshewski, A.; Lachner, N.; Ouwerkerk, D.; Klieve, A.V.; Dart, P.; Hugenholtz, P. Near complete genome sequence of the animal feed probiotic, *Bacillus amyloliquefaciens* H57. *Stand. Genom. Sci.* **2016**, *11*, 60. [CrossRef]
19. Cawoy, H.; Mariutto, M.; Henry, G.; Fisher, C.; Vasilyeva, N.; Thonart, P.; Dommes, J.; Ongena, M. Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. *Mol. Plant Microbe Interact.* **2014**, *27*, 87–100. [CrossRef]
20. Han, Q.; Wu, F.L.; Wang, X.N.; Qi, H.; Shi, L.; Ren, A.; Liu, Q.H.; Zhao, M.W.; Tang, C.M. The bacterial lipopeptide iturins induce *Verticillium dahliae* cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen-associated molecular pattern-triggered immunity. *Environ. Microbiol.* **2015**, *17*, 1166–1188. [CrossRef]
21. Farzaneh, M.; Shi, Z.Q.; Ahmadzadeh, M.; Hu, L.B.; Ghassempour, A. Inhibition of the *Aspergillus flavus* growth and *Aflatoxin* B1 contamination on pistachio nut by fengycin and surfactin-producing *Bacillus subtilis* UTBSP1. *Plant Pathol. J.* **2016**, *32*, 209–215. [CrossRef] [PubMed]
22. Leclère, V.; Béchet, M.; Adam, A.; Guez, J.S.; Wathelet, B.; Ongena, M.; Thonart, P.; Gancel, F.; Chollet-Imbert, M.; Jacques, P. Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Appl. Environ. Microb.* **2005**, *71*, 4577–4584. [CrossRef] [PubMed]
23. Ni, M.; Wu, Q.; Wang, J.L.; Liu, W.C.; Ren, J.H.; Zhang, D.P.; Zhao, J.; Liu, D.W.; Rao, Y.H.; Lu, C.G. Identification and comprehensive evaluation of a novel biocontrol agent *Bacillus atrophaeus* JZB120050. *J. Environ. Sci. Health B* **2018**, *53*, 777–785. [CrossRef]
24. Pei, J.J.; Yue, T.L.; Jin, W.G. Application of bacteriocin RC20975 in apple juice. *Food Sci. Technol. Int.* **2016**, *23*, 166–173. [CrossRef]
25. Chowdhury, S.P.; Hartmann, A.; Gao, X.W.; Borriss, R. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42-a review. *Front. Microbiol.* **2015**, *6*, 780. [CrossRef] [PubMed]
26. Muñoz, C.Y.; Zhou, L.; Yi, Y.H.; Kuipers, O.P. Biocontrol properties from phyllospheric bacteria isolated from *Solanum lycopersicum* and *Lactuca sativa* and genome mining of antimicrobial gene clusters. *BMC Genom.* **2022**, *23*, 152. [CrossRef]
27. Li, L.; Yang, B.L.; Humza, M.; Geng, H.R.; Wang, G.; Zhang, C.X.; Gao, S.; Xing, F.G.; Liu, Y. A novel strain *Lactobacillus brevis* 8-2B inhibiting *Aspergillus carbonarius* growth and ochratoxin a production. *LWT* **2021**, *136*, 110308. [CrossRef]
28. Khan, M.S.; Gao, J.L.; Zhang, M.F.; Xue, J.; Zhang, X.H. *Pseudomonas aeruginosa* Ld-08 isolated from *Lilium davidii* exhibits antifungal and growth-promoting properties. *PLoS ONE* **2022**, *17*, e0269640. [CrossRef]
29. Dong, X.Z.; Cai, M.Y.B. *Systematic Determinative Manual of General Bacteria*; Science Press: Beijing, China, 2001; pp. 353–370.
30. Wang, B.B.; Yuan, J.; Zhang, J.; Shen, Z.Z.; Zhang, M.X.; Li, R.; Ruan, Y.Z.; Shen, Q.R. Effects of novel bioorganic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of *Fusarium* wilt of banana. *Biol. Fertil. Soils* **2013**, *49*, 435–446. [CrossRef]
31. Chun, J.; Bae, K.S. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. *Antonie Van Leeuwenhoek* **2000**, *78*, 123–127. [CrossRef]
32. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
33. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
34. Wang, L.M.; Xi, N.; Lang, D.Y.; Zhou, L.; Zhang, Y.J.; Zhang, X.H. Potential biocontrol and plant growth promotion of an endophytic bacteria isolated from *Glycyrrhiza uralensis* seeds. *Egypt. J. Biol. Pest Control* **2022**, *32*, 55. [CrossRef]
35. Li, E.F.; Wang, G.; Xiao, J.L.; Ling, J.; Yang, Y.H.; Xie, B.Y. A SIX1 homolog in *Fusarium oxysporum* f. sp. *conglutinans* is required for full virulence on cabbage. *PLoS ONE* **2016**, *11*, 0152273.
36. Jing, C.L.; Zhao, J.; Han, X.B.; Huang, R.H.; Cai, D.S.; Zhang, C.S. Essential oil of Syringa oblata Lindl. As a potential biocontrol agent against tobacco brown spot caused by *Alternaria alternata*. *Crop Prot.* **2018**, *104*, 41–46. [CrossRef]
37. Ding, T.; Su, B.; Chen, X.J.; Xie, S.S.; Gu, S.Y.; Wang, Q.; Huang, D.Y.; Jiang, H.Y. An endophytic bacterial strain isolated from *Eucommia ulmoides* inhibits Southern corn leaf blight. *Front. Microbiol.* **2017**, *8*, 903. [CrossRef] [PubMed]
38. Finkel, O.M.; Castrillo, G.; Paredes, S.H.; González, I.S.; Dangl, J.L. Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* **2017**, *38*, 155–163. [CrossRef]
39. Liu, Z.P.; Wang, H.X.; Xu, W.H.; Wang, Z.G. Isolation and evaluation of the plant growth promoting rhizobacterium *Bacillus methylotrophicus* (DD-1) for growth enhancement of rice seedling. *Arch. Microbiol.* **2020**, *202*, 2169–2179. [CrossRef]
40. Liu, X.M.; Li, Q.; Li, Y.B.; Guan, G.H.; Chen, S.F. *Paenibacillus* strains with nitrogen fixation and multiple beneficial properties for promoting plant growth. *PeerJ* **2019**, *7*, e7445. [CrossRef]

41. Chen, Y.P.; Rekha, P.D.; Arun, A.B.; Shen, F.T.; Lai, W.A.; Young, C.C. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Appl. Soil Ecol.* **2006**, *34*, 33–41. [CrossRef]
42. Zhou, H.; Ren, Z.H.; Zu, X.; Yu, X.Y.; Zhu, H.J.; Li, X.J.; Zhong, J.; Liu, E.M. Efficacy of plant growth-promoting bacteria *Bacillus cereus* YN917 for biocontrol of rice blast. *Front. Microbiol.* **2021**, *12*, 684888. [CrossRef] [PubMed]
43. Zhai, Y.; Zhu, J.X.; Tan, T.M.; Xu, J.P.; Shen, A.R.; Yang, X.B.; Li, J.L.; Zeng, L.B.; Wei, L. Isolation and characterization of antagonistic *Paenibacillus polymyxa* HX-140 and its biocontrol potential against Fusarium wilt of cucumber seedlings. *BMC Microbiol.* **2021**, *21*, 75. [CrossRef] [PubMed]
44. Chen, X.H.; Koumoutsi, A.; Scholz, R.; Schneider, K.; Vater, J.; Süssmuth, R.; Piel, J.; Borriss, R. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *J. Biotechnol.* **2009**, *140*, 27–37. [CrossRef] [PubMed]
45. Selim, H.M.M.; Gomaa, N.M.; Essa, A.M.M. Application of endophytic bacteria for the biocontrol of *Rhizoctonia solani* (Cantharellales: Ceratobasidiaceae) damping-off disease in cotton seedlings. *Biocontrol Sci. Technol.* **2017**, *27*, 81–95. [CrossRef]
46. Beauregard, P.B. Chapter One—Not just sweet talkers: How roots stimulate their colonization by beneficial bacteria. *Adv. Bot. Res.* **2015**, *75*, 1–20.
47. Raaijmakers, J.M.; Bruijn, I.D.; Nybroe, O.; Ongena, M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: More than surfactants and antibiotics. *FEMS Microbiol. Rev.* **2010**, *346*, 1037–1062. [CrossRef]
48. Falardeau, J.; Wise, C.; Novitsky, L.; Avis, T.J. Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. *J. Chem. Ecol.* **2013**, *39*, 869–878. [CrossRef]
49. Coy, R.M.; Held, D.W.; Kloepper, J.W. Rhizobacterial colonization of bermudagrass by *Bacillus* spp. in a Marvyn loamy sand soil. *Appl. Soil Ecol.* **2019**, *141*, 10–17. [CrossRef]
50. Walker, T.S.; Bais, H.P.; Grotewold, E.; Vivanco, J.M. Root exudation and rhizosphere biology. *Plant Physiol.* **2003**, *132*, 44–51. [CrossRef]
51. Steinkellner, S.; Mammerler, R.; Vierheilig, H. Germination of *Fusarium oxysporum* in root exudates from tomato plants challenged with different *Fusarium oxysporum* strains. *Eur. J. Plant Pathol.* **2008**, *122*, 398–401. [CrossRef]
52. Fan, B.; Borriss, R.; Bleiss, W.; Wu, X.Q. Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. *J. Microbiol.* **2012**, *50*, 38–44. [CrossRef] [PubMed]
53. Pandic, C.; Coq, D.L.; Deschamps, J.; Védie, R.; Rousseau, T.; Aymerich, S.; Briandet, R. Complete genome sequence of *Bacillus velezensis* QST713: A biocontrol agent that protects *Agaricus bisporus* crops against the green mould disease. *J. Biotechnol.* **2018**, *278*, 10–19. [CrossRef] [PubMed]
54. Blom, J.; Rueckert, C.; Niu, B.; Wang, Q.; Borriss, R. The complete genome of *Bacillus amyloliquefaciens* subsp. *plantarum* CAU B946 contains a gene cluster for nonribosomal synthesis of Iturin A. *J. Bacteriol.* **2012**, *194*, 1845–1846. [PubMed]
55. Posada, L.F.; Álvarez, J.C.; Romero-Tabarez, M.; de-Bashan, L.; Villegas-Escobar, V. Enhanced molecular visualization of root colonization and growth promotion by *Bacillus subtilis* EA-CB0575 in different growth systems. *Microbiol. Res.* **2018**, *217*, 69–80. [CrossRef]