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Evaluating the Efficacy of the Fermentation Formula of *Bacillus velezensis* Strain Tcb43 in Controlling Cucumber Powdery Mildew

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Abstract: Powdery mildew is a prevalent disease that affects cucumber crops, leading to yield reductions and declines in quality. *Bacillus* sp. strain Tcb43 culture medium was shown to be capable of inhibiting fungal spore germination in previous reports. In this study, the identification of the Tcb43 strain was updated to *Bacillus velezensis* by using whole-genome sequencing. The strain exhibited tolerance to a wide range of temperatures (12–40 °C), salinities (7–10%), and pH levels (ranging from 5 to 11). Additionally, Tcb43 demonstrated insensitivity to most tested fungicides. A new fermentation formula, Tcb43FBSO, was developed by adding 0.25% soybean oil to the fermented formula (Tcb43FB). This new formula exhibited a shelf life of up to 12 months with the decrease in bacterial count from 5.35×10^8 to 1.97×10^8 cfu/mL. Greenhouse assays showed that the treatment of potted cucumber plants with a 100-fold dilution (100×) of Tcb43FBSO for four weeks resulted in a significant reduction (64.64%) of cucumber powdery mildew compared to the mock group. In large-scale greenhouse trials, the treatment of cucumber plants with 200× of Tcb43FBSO for 5 weeks effectively suppressed powdery mildew disease, with a control rate that reached 76.6% compared to the mock group. These findings highlight the potential of Tcb43 as a biocontrol agent for managing cucumber powdery mildew and suggest its promising application in agriculture.

Keywords: *Podosphaera xanthii*; biocontrol; oil addition; fermentation

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1. Introduction

Cucumber (*Cucumis sativus*) is one of the most important vegetable crops in Taiwan. The cultivation area of cucumber is approximately 1949 hectares, with about 47,975 tons being produced in 2021 [1,2]. Cucumber powdery mildew is known to be caused by several pathogens, such as *Podosphaera xanthii*, *Golovinomyces cucurbitacearum*, *G. orontii*, and *Leveillula Taurica* [3]. There are more than 200 species of powdery mildew hosts in Taiwan, but the pathogens are different and specific. Among the economically important crops affected are melons, members of the Solanaceae family, leguminous crops, and various fruit trees, among others [4]. Cucumber powdery mildew is an important leaf disease and seriously affects yields. In Taiwan, cucumber powdery mildew is mainly due to *P. xanthii* [1]. Most cucumbers are cultivated in central Taiwan in large greenhouses to avoid damage from insect pests. However, poor ventilation and high humidity in greenhouses may lead to the rapid development of powdery mildew disease [1]. When the disease is severe, the pathogen can infect leaves, branches, and fruits during all growth stages of the plant. The mycelium and conidia of *P. xanthii* cover heavily infected tissues throughout the plant surface, reducing photosynthesis and resulting in lower yields and fruit quality [5]. Chemically synthesized fungicides have successfully controlled powdery mildew, but fungicides pose health problems and cause environmental pollution [6]. Therefore,

alternative strategies are needed to control this disease by applying resistance-inducing chemicals, natural products, oils or biological agents [6–10]. To date, except for chemical agents and registered plant protection control materials, no microbial agent has been approved for the control of powdery mildew in cucumber plants in Taiwan. The development of biological agents is a trend in the control of powdery mildew in various countries [9]. To develop biocontrol agents, *Bacillus amyloliquefaciens* LJ02 was applied in greenhouse trials in China, showing that LJ02 fermentation broth (LJ02FB) can effectively diminish the occurrence of cucurbits powdery mildew in greenhouse experiments [11]. In Korea, *B. amyloliquefaciens* M27 was found to be potent against cucumber powdery mildew on cucumber leaves, showing a 4.0% reduction in greenhouse tests [12]. Under greenhouse conditions, it has been reported that spraying cell suspensions or culture filtrates of *Bacillus subtilis*, *B. licheniformis*, *B. aerius*, *Pseudomonas fluorescens*, *Derxia gummosa*, *Trichoderma harzianum,* or *T. asperellum* onto cucumber plants significantly reduced the severity of powdery mildew on cucumber and increased fruit yield [13–15]. In addition, Canadian greenhouses have access to some registered commercial biological control products, such as *B. subtilis* QST 713 (sold as Rhapsody® and Serenade®), *Gliocladium* catenulatum strain J1446 (marketed as Prestop®), *Streptomyces lydicus* WYEC 108 (available as Actinovate®), *Streptomyces* griseoviridis K61 (sold as Mycostop®), and *T. harzianum* strain T-22 (marketed as Rootshield®). Within the greenhouse industry, the use of bioagents has emerged as a valuable and effective strategy for biological control [9]. These bioagents employ various mechanisms, including antibiosis, competition, mycoparasitism, and induced resistance, to effectively control phytopathogenic fungi [15]. These approaches offer viable options for promoting sustainable and environmentally friendly practices in greenhouse cultivation.

Recent studies have demonstrated that certain vegetable oils, including rapeseed oil, babassu oil, corn oil, sunflower oil, or soybean oil, are cost-effective raw materials that can effectively induce microbial surfactant production, e.g., rhamnolipid, sophorolipid, and mannose erythritol lipid biosurfactants [16]. Biosurfactants can reduce surface and interfacial tension, promote foaming, stabilize emulsions, and maintain specific activity under extreme pH values, temperatures, and salinity conditions [17]. In the field of agriculture, microorganisms capable of producing biosurfactants may serve as a biological agents against phytopathogens [18,19]. For instance, *Bacillus* sp. A5F fermented using glucose and soybean oil resulted in the optimal production of biosurfactants. These biosurfactants had a significant positive impact on shoot biomass, pod number, seed weight, and chlorophyll content. Moreover, the biosurfactants also exhibited disease-suppressing properties against *Sclerotinia sclerotiorum* by disrupting the hyphal cell wall [20].

In our previous study, the beneficial microorganism Tcb43 strain was isolated from the soil of an organic field where red dragon fruit was cultivated [21]. Through the use of *16S rRNA* and *gyrB* gene sequencing, the Tcb43 strain was identified as *B. amyloliquefaciens* [21]. The Tcb43 strain exhibited four types of decomposing enzyme activities of starch, lipid, cellulose, and protein. In addition, the strain Tcb43 showed antagonistic activity against the pathogens of cucumber anthracnose, cucumber brown spot, cucumber wilt, pepper anthracnose, grape ripe rot, and muskmelon root rot/vine decline in in vitro assays [21]. The Tcb43 strain demonstrated inhibitory effects on the conidial germination of powdery mildew of cucumber, melon, and sweet pea, with inhibition rates exceeding 98.25%, 85.96%, and 93.96%, respectively. These results showed that the Tcb43 strain has the ability to inhibit pathogenic fungi and could be developed as a microbial pesticide. Consequently, we aimed to evaluate the fermentation and formulation of Tcb43 in large-scale greenhouse trials. In this study, we sequenced the whole genome of Tcb43 to confirm its identity as *Bacillus velezensis* (Bv). Physiological tests indicated that Tcb43 could tolerate adverse conditions such as salinity, temperature, pH value, and pesticides commonly used to control phytophthora rot, powdery mildew, downy mildew, or rhizoctonia seedling blight diseases on cucumber plants. We conducted experiments to assess the shelf-life stability and long-term storage of a Tcb43 fermentation broth (Tcb43FB) formulated with soybean oil (referred to as Tcb43FBSO). The addition of soybean oil not only improved the efficiency

of Tcb43 control trials in potted cucumber plants and large-scale greenhouses but also contributed to the stability of the formulation. In the potted plants assay, the control rate of 100-fold dilution (100×) of Tcb43FB and 100× of Tcb43FBSO was 41.63% and 64.64%, respectively, and the control rate of 200× of Tcb43FBSO reached approximately 76.6% in three large-scale trials. Our findings provide evidence that Bv Tcb43 exhibits great potential as an effective biocontrol agent against powdery mildew diseases. This promising candidate demonstrates favorable traits in terms of environmental friendliness and disease control for future development in this field.

2. Materials and Methods

2.1. Bacterial Strains and Media

The Tcb43 strain was stored at −80 °C in 20% (*v*/*v*) glycerol. The Tcb43 strain was grown on nutrient agar (NA) or Luria–Bertani (LB) agar. After culturing in a 30 °C incubator for 48 h (hrs), a single colony was picked and subcultured on NA. After two generations, a single colony was picked and cultured in 50 mL of LB Broth. The culture was shaken at 150 rpm at 30 °C for 48 h as a fermentation inoculum.

2.2. Characterization and Identification of the Tcb43 Strain

2.2.1. Identification of Whole-Genomic DNA Sequencing

The biomass of the Tcb43 strain was obtained after growth under optimal conditions. Genomic DNA was extracted using the phenol/chloroform method as described [22]. Gel electrophoresis and NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) were used to assess the purity of the DNA. Whole-genome sequencing was performed using the Illumina and Nanopore sequencing platform (Inong Agriculture Co., Ltd., Taiwan). The sequence contigs were assembled using SPAdes v3.15.4 (https://github.com/ablab/spades), and Prokka v1.13 accessed on 11 April 2022 (https://github.com/tseemann/prokka) was utilized as a gene prediction tool. The genomics analysis was conducted by Genomics BioSci & Tech Co., Ltd. (New Taipei City, Taiwan). OrthoANI was used to analyze the evolution relationship of the Tcb43 strain [23,24].

2.2.2. Sensitivity to Different Stressors

Tcb43 was grown on NA amended with different concentrations of 7%, 8%, or 10% NaCl to assess its salt tolerance. To test pH tolerance, Tcb43 was cultivated on NA with pH levels ranging from 5 to 11. To test temperature tolerance, Tcb43 culture plates were subjected to a range of temperatures, including 8 °C, 12 °C, 16 °C, 20 °C, 24 °C,28 °C, 32 °C, 37 °C, and 40 °C. To assess susceptibility to fungicides, Tcb43 was grown on NA amended with various chemicals, which were recommended for controlling powdery mildew, downy mildew, phytophthora rot, or rhizoctonia seedling blight diseases in cucumber plants [25]. The test chemicals included Etridiazole + hymexazol wettable powder (WP) (Great Victory Chemical Industry Co., Yunlin, Taiwan) at 0.03% (*w*/*w*), Propamocarb hydrochloride soluble concentrate (SL) (Taiwan Scientific Biotechnology Co., Taichung, Taiwan) at 0.166% (*w*/*w*), Cyazofamid suspension concentrate (SC) (Ya Chung Industrial Co., Taipei, Taiwan) at 0.003% (*w*/*w*), Etridiazole emulsifiable concentrate (EC) (Great Victory Chemical Industry Co.) at 0.017(*w*/*w*), Azoxystrobin SC (Chia Yi Chemical Industry Co., Changhua, Taiwan) at 0.023 (*w*/*w*), Dimethomorph WP (BASF Taiwan, Taipei, Taiwan) at 0.017 (*w*/*w*), Fluopicolide + Propamocarb hydrochloride SC (Bayer Taiwan Co., Taipei, Taiwan) at 0.0608 (*w*/*w*), Metiram water-dispersible granules (WG) (BASF Taiwan, Taipei, Taiwan) at 0.16 (*w*/*w*), Kasugamycin + Copper oxychloride WP (Great Victory Chemical Industry Co., Yunlin, Taiwan) at 0.0775 (*w*/*w*), Ametoctradin + Dimethomorph SC (BASF Taiwan, Taipei, Taiwan) at 0.0525 (*w*/*w*), Mancozeb + Zoxamide WG (Lih-Nung Chemical Co., Yunlin, Taiwan) at 0.075 (*w*/*w*), mancozeb + metalaxyl WP (Huikwang Co., Tainan, Taiwan) at 0.145 (*w*/*w*), Chlorothalonil WP (Huikwang Co., Tainan, Taiwan) at 0.15 (*w*/*w*), Polyoxins water-soluble granule (SG) (Sinon Corporation, Taichung, Taiwan) at 0.01 (*w*/*w*), Sulfur SC (Moralburg Agro. International Corporation, Taichung, Taiwan) at 0.08 (*w*/*w*), 0.0125 (*w*/*w*)

Fluopyram+ Trifloxystrobin SC (Bayer Taiwan Co., Taipei, Taiwan), Penconazole Emulsion, oil in water (EW) (Rich Country Chemical Corporation, Taipei, Taiwan) at 0.003 (*w*/*w*), Bupirimate EC (Lanlix Crop Science Co., Pingtung, Taiwan) at 0.008 (*w*/*w*), Procymidone WP (Lih-Nung Chemical Co., Yunlin, Taiwan) at 0.025 (*w*/*w*), and thiophanate-methyl WP (Tai Yeh Chemical Industry co., Hsinchu, Taiwan) at 0.07 (*w*/*w*).

All fungicides were applied following the guidelines provided by the manufacturer. To assess the sensitivity of Tcb43 to compound effectiveness, Tcb43 was incubated at 28 °C for 24 h.

2.3. Formulation of the Fermentation Broth

Tcb43 was cultured in 50 mL of LB broth with constant shaking in a 30 °C incubator for 48 hrs and then collected as a mother liquor for a bench-scale 10 L fermenter system (WinPact Parallel Fermentation System FS-05, Major Science Co., LTD., Taoyuan, Taiwan). In the fermentation process of the 10 L fermenter, the basic formula (0.25% yeast extract (Ensenmi Bio-Technology Co., Ltd., Guangdong, China), 0.1% dipotassium hydrogen phosphate (Union Chemical Works Ltd., Kaohsiung, Taiwan), 0.1% magnesium sulfate (Honeywell Fluka, Seelze, Germany), and 0.1% manganese sulphate (Echo chemical Co., Ltd. Miaoli, Taiwan)) were used as the main culture substrate to search for the suitable formula and proportion. After incubation, samples were regularly taken to determine the bacterial concentration and endospore conversion rate, and when the Dissolved Oxygen (DO) in the fermenter was close to 100%, the fermentation broth was collected.

2.3.1. Optimal Carbon–Nitrogen Ratio Optimization in Fermentation Broth

In order to test the appropriate carbon–nitrogen ratio of the basic formulation of the Tcb43 fermentation broth, No. 2 granulated sugar (Taiwan sugar corp., Tainan, Taiwan) was used as the carbon source, and soybean protein (Soya product, Sonic Biochem Extractions Pvt Ltd., Madhya Pradesh, India) was used as the nitrogen source. Carbon and nitrogen sources were cross-added at 1%, 2%, and 3% (*w*/*w*), and Tcb43 was cultured in a 500 mL Erlenmeyer flask at 30 °C, with constant shaking at 120 rpm (Model: S300R, Firstek scientific Co., Ltd., New Taipei City, Taiwan). The samples were taken every 24 h after inoculation to detect the number of viable Tcb43 bacteria.

2.3.2. Effect of Vegetable Oil in Tcb43 Population in Tcb43 Fermentation Broth

The effect of different vegetable oils on the Tcb43 population was determined in a fermentation broth mixed with various commercial vegetable oils, and after cultivation, Tcb43 cell counts were determined via dilution plating. Then, 1% of different vegetable oils, such as sunflower oil (Great Day, Standard Foods Co., Taipei, Taiwan), avocado oil (Chosen Foods, Hancock St Ste A, San Diego, CA, USA), soybean oil (Fwusow industry Co., Ltd., Taichung, Taiwan), citronella oil (United Pros Corp., Taipei, Taiwan), wintergreen oil (Cheng J Chemical Material Co., Ltd., Taipei, Taiwan), and olive oil (Taisun Enterprise Co., Ltd., Changhua, Taiwan), were selected and each mixed with 10% of tween 80 (Sigma) as an emulsifier to a final concentration of 1% (*v*/*v*) in fermented Tcb43 broth. After incubating the mixture at room temperature for 24 h, the Tcb43 colony counts on plates were enumerated.

2.3.3. Optimization of Soybean Oil Concentration in Tcb43 Fermentation Formulation

Tcb43 fermentation broth formula containing 0.25%, 0.5%, and 1% soybean oil were selected for simultaneous fermentation for 3 days, and the Tcb43 population was counted every 24 h for 3 days, whereas Tcb43 cultured in LB was counted and used as controls.

2.4. Sporulation and Shelf-Life Detection of Tcb43 in Fermentation Broth

The shelf lives of the fermented broth (Tcb43FB) and the Tcb43FB amended with 0.25% soybean oil (Tcb43FBSO) were evaluated. These formulations were fermented in a 10 L fermentation system for 60 h at 28 °C, with a rotational speed of 400 rpm and normal

aeration volume (100 LPM). The sporulation ratio of Tcb43 was determined as the number of spores divided by the number of viable cells.

After fermentation, Tcb43FB and the Tcb43FBSO were stored at 25 °C, and an aliquot of 10 mL from the fermented broths were taken and checked for endospores every two months a total of 6 times. The broths were boiled at 60 °C for 30 min to lyse the vegetative cells before the endospore numbers of the tested fermented broths were counted.

2.5. Potted-Plant Assay for Control Powdery Mildew Disease Control

The experiment was carried out in a simple net room at the Taichung Agricultural Improvement Farm (Taichung District). The tested cucumber varieties, cucumber seeds cv. Known-You variety Cuigu with the product number HV-264 (Known-You Seed Co., Kaohsiung, Taiwan) were planted in peat moss (BVB no. 4, Bas Van Buuren, Maasland, New Zealand) within a seedling tray. The seedlings were cultivated in a greenhouse under controlled conditions, with a daily cycle of 16 h of light at 28 °C and 8 h of darkness at 25 °C for approximately 2–3 weeks before being transplanted to 3-inch pots. Each treatment consisted of 8 potted cucumber plants, which were (1) mock, (2) 100-fold dilution (100×) of Tcb43 cultured in LB, (3) 100× of Tcb43 fermentation broth (Tcb43FB), and (4) 100× of Tcb43 supplemented with 0.25% soybean oil (Fwusow industry Co., Ltd., Taichung, Taiwan) fermentation broth (Tcb43FBSO). After sporadic powdery mildew lesions appeared naturally on the cucumber leaves, the first spray was carried out for each treatment, and the same applications were continuously performed every 7 days 4 times. The disease incidence of powdery mildew in cucumber plant was investigated and recorded weekly before each spray. Disease rating was performed using a scale ranging from 0 to 4 for assessing the disease severity: 0, no symptoms; level 1, powdery mildew leaf area is about 1–10%; level 2, diseased leaf area is about 10.1–15%; level 3, diseased leaf area is about 15.1–25%; level 4, diseased leaf area is about 25.1–50%; and level 5, diseased leaf area is 50.1–100%. The disease rating was calculated using the following formula: Disease severity (%) = [Σ (scale value × number of infected plants at each scale value)/total number of plants × 5] × 100% [26]. The disease control rate was calculated using the following formula: (%) = [1 − (Disease severity of the treatment group/Disease severity of the control group)] × 100%. The individual experiments were repeated four times.

2.6. Large-Scale Greenhouse Trials

From 2020 to 2023, Tcb43 fermentation broth was utilized for powdery mildew control in the organic cucumber field in a large-scale greenhouse in Taichung District Agricultural Research and Extension Station in Taichung District. The field trial experiment followed a randomized complete block design (RCBD) [27]. Each treatment consisted of 8 cucumber plants (variety Cuigu, Known-You), with 4 repetitions, resulting in a total of 16 randomly arranged plots in the test area. The plants were grown directly on the soil for 2–3 weeks in a greenhouse. Once the occurrence of powdery mildew lesions was observed, treatment with the Tcb43 fermentation broth commenced. Four different treatments were applied, including group 1: 200-fold dilution (200×) of Tcb43FB + 0.25% Soybean oil (Tcb43FBSO); group 2: 400-fold dilution (400×) of Tcb43FBSO; group 3: 600-fold dilution (600×) of Tcb43FBSO; and group 4: water (mock control). Then, 200 mL of the treatment broth was sprayed onto each plant. Treatments were performed every 7 days with 5 repeats. Disease rating was conducted on 8 plants per lane, and disease incidence was determined using 10 leaves of each plant from top to bottom. The disease severity (%) was calculated as described above. The area under the disease progress curve (AUDPC) was calculated as $\sum_{i=1}^{n-1} \frac{(y_i+y_{i+1})(t_{i+1}-t_i)}{2}$, where yi = disease severity at time i; $t_{i+1} - t_i$ = day interval between two ratings; n = number of ratings [28]. The individual experiments were repeated three times.

2.7. Statistical Analysis

The quantitative data were subjected to a statistical test to assess the homogeneity of variance and were analyzed for variance using the IPM SPSS statistics software Version 20 (IBM Corp., Armonk, NY, USA). To examine the impact of different treatments in all assays, a one-way analysis of variance (ANOVA) was employed. Differences between treatments were determined through Tukey's honestly significant difference (HSD) analysis or Student's *t*-test ($p < 0.05$).

3. Results

3.1. Confirmation and Classification of Tcb43 Strain as Bacillus velezensis through Whole-Genome Sequencing

To accurately differentiate between *B. amyloliquefaciens* and *B. velezensis* strains, the *16S rRNA* gene and *gyrB* gene sequences alone were insufficient [29]. Therefore, we conducted whole-genome sequencing for the Tcb43 strain. According to the sequencing results, the genome size of Tcb43 (accession number JAQNDE000000000) was 3.98 Mb, with a G+C content 46.37%. To determine the taxonomic classification, the OrthoANI software utilized the 95% average nucleotide identity (ANI) value as the species boundary for calculation [23,24]. Therefore, based on the analysis, the genome of Tcb43 was similar to that of the *B. velezensis* FZB42 strain (PRJNA277720) with 98.62% similarity, and it was also similar to the *B. velezensis* strain SRCM103616 (PRJNA516721 with 97.89% similarity). These findings confirm that the Tcb43 strain belongs to the *Bacillus velezensis* species.

3.2. Sensitivity to Stressors

Stress tests revealed the ability of Tcb43 to thrive across a wide pH range from 5 to 11. The strain can also tolerate salt concentrations ranging from 7% to 10% and temperatures between 12 and 40 °C. Regarding chemical assays, the growth of Tcb43 was unaffected by most tested fungicides, including Etridiazole + hymexazol, Propamocarb hydrochloride, Cyazofamid, Etridiazole Emulsifiable, Azoxystrobin, Dimethomorph, Fluopicolide + Propamocarb, Ametoctradin + Dimethomorph, Polyoxins water-soluble granule, Sulfur, Fluopyram + Trifloxystrobin, Procymidone, and thiophanate-methyl at the test concentrations. However, Tcb43 was sensitive to certain fungicides, namely Metiram water-dispersible granules, Kasugamycin + Copper oxychloride WP, Mancozeb + Zoxamide WG, mancozeb + metalaxyl WP, Chlorothalonil WP, Penconazole Emulsion, and Bupirimate EC.

3.3. Formulation of the Tcb43 Biocontrol Agent

3.3.1. The Optimal Carbon–Nitrogen Ratio for Tcb43 Fermentation Broth

The test for the optimal carbon–nitrogen ratio for the Tcb43 strain fermentation broth showed that Tcb43 reached the highest population at 6.65×10^8 cfu/mL when 3% carbon (in the form of No. 2 granulated sugar) and 3% nitrogen (provided by soybean protein) were added to the basic culture components (0.25% yeast extract, 0.1% dipotassium hydrogen phosphate, and 0.1% magnesium sulfate) on the third day of cultivation (Figure 1). Hence, taking into consideration the sufficient time required for the vegetative cells of Tcb43 to transition into endospores during the lag phase, the Tcb43 fermentation formula (Tcb43FB) with a 3-day culture period was adopted for subsequent experiments.

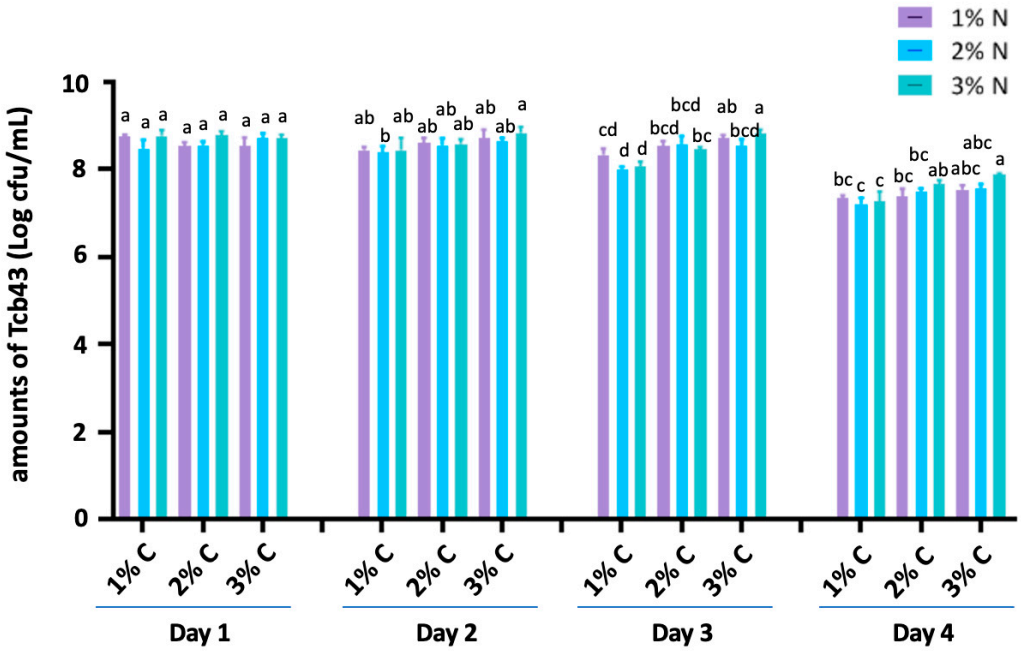

Figure 1. The optimal carbon–nitrogen ratio of *Bacillus velezensis* Tcb43 fermentation broth. The culture experiment spanned 1–4 days of fermentation, with varying concentrations of No. 2 sugar (1–3%) and soybean protein (1–3%). Statistical analysis using Tukey's HSD test ($p < 0.05$, n = 3) on the daily culture counts demonstrated significant difference between means, denoted by distinct letters (a, b, c or d) within the columns.

3.3.2. Influence of Different Vegetable Oil on Tcb43 Population in Fermentation Broth

In order to test whether commercially available vegetable oils as surfactants affected the bacterial population in the Tcb43 fermentation broth, various oils such as sunflower oil, avocado oil, soybean oil, citronella oil, wintergreen oil, and olive oil were mixed into the fermentation broth, and the bacterial count was observed one day after mixing. Surprisingly, none of the test vegetable oils showed any significant effect on the Tcb43 bacterial population. The bacterial count remained consistently high, with a logarithmic value ranging from approximately 7.84 to 8.0, regardless of the oil used. Considering cost-effectiveness, soybean oil was chosen for all subsequent experiments.

3.3.3. The Optimal Concentration of Soybean Oil in Tcb43 Fermentation

In order to test the effect of different concentrations of soybean oil in Tcb43 formulation, Tcb43 was fermented for three days and the bacterial count was monitored daily. A comparison was made among Tcb43 grown on LB broth (Tcb43LB); the fermentation broth (Tcb43FB); and fermentation broth with 0.25%, 0.5%, or 1.0% soybean oil added. The results showed that after 3 days of fermentation in Tcb43FB supplemented with soybean oil, the bacterial counts were significantly higher than Tcb43LB. Among the tested concentrations, the supplementation of 0.25% soybean oil yielded the highest average bacterial count: approximately 8.59×10^8 cfu/mL (Figure 2). Subsequently, Tcb43FB or the addition of 0.25% soybean oil was adopted for subsequent fermentation formulas to evaluate the shelf life and long-term storage capabilities.

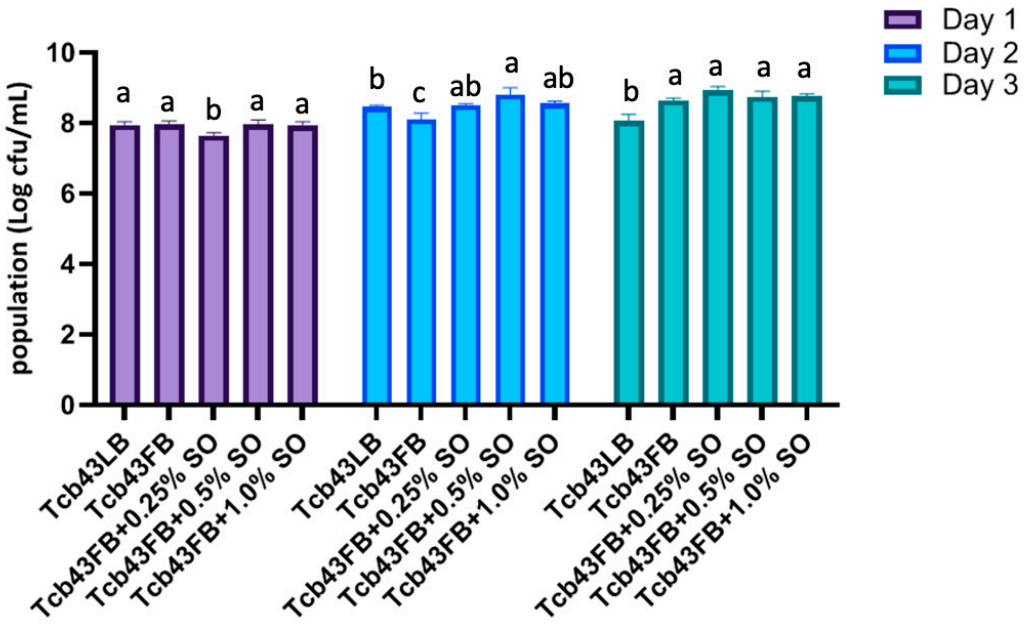

Figure 2. The comparison of *Bacillus velezensis* Tcb43 fermentation formulas with varying concentrations of soybean oil. Tcb43 cultured in LB medium (Tcb43LB), fermentation broth formula (Tcb43FB), and fermented formula with 0.25% soybean oil (Tcb43FB + 0.25%SO), 0.5% soybean oil (Tcb43FB + 0.5%SO), and 1.0% soybean oil (Tcb43FB + 1.0%SO) added to observe the bacterial count for 1–3 days after fermentation. Statistical analysis using Tukey's HSD test ($p < 0.05$, n = 3) indicated significant differences between means, represented by distinct letters (a, b, or c) within the columns.

3.4. Sporulation and Shelf Life Tests of Tcb43 Fermentation Broth

The comparison of the shelf life of the fermented broth (Tcb43FB) and Tcb43FB supplemented with 0.25% soybean oil (Tcb43FBSO), after 60 h of fermentation in a 10 L fermentation tank, shows a high viable bacterial count in Tcb43FB (2.7×10^8 cfu/mL). In addition, the number of treated endospores after 60 °C heating reached 8.67×10^7 cfu/mL, translating to a ~32% sporulation ratio. On the other hand, Tcb43FBSO resulted in a higher bacterial viability of ~8.25×10^8 cfu/mL and an endospore count of ~5.35×10^8 cfu/mL, resulting in a sporulation ratio of ~65%.

The shelf lives of both fermentation formulas were monitored by counting the endospores that germinated every two months for twelve months. The results revealed a sharp decline in the endospore content of Tcb43FB after two months, with only 1.67×10^6 cfu/mL remaining after twelve months (Figure 3). Conversely, the endospore content in Tcb43FBSO showed no significant change during each two-month sampling period relived to the initial spore content. After 12 months, the average endospore content in Tcb43FBSO remained at 1.97×10^8 cfu/mL. These findings demonstrate that Tcb43FBSO exhibited a superior shelf life compared to Tcb43FB (Figure 3).

3.5. Evaluation of Tcb43 Fermentation Formula for Controlling Powdery Mildew Disease in Potted Plants

To assess the efficacy of the Tcb43 fermentation formula in controlling cucumber powdery mildew disease, a greenhouse experiment was conducted. The results showed that three weeks after application, both the 100-fold dilution (100×) of Tcb43 fermentation broth (Tcb43FB) and 100× of Tcb43FB supplemented with soybean oil (Tcb43FBSO) significantly reduced the severity of powdery mildew compared to the mock treatment or 100× of Tcb43 cultured in LB (Tcb43LB) (Figure 4). This ability of the formulation to control the powdery mildew effect persisted until the fourth week after application. The disease severity of powdery mildew in four treatments was 57.90% (mock), 40.00% (Tcb43LB), 33.80% (Tcb43FB), and 20.48% (Tcb43FBSO), respectively. Moreover, the disease control

rate of 100× of Tcb43FB and Tcb43FBSO was 41.63% and 64.64%, respectively. Visual observation of the potted plants also revealed noticeable differences in disease symptoms on cucumber leaves among different treatments (Figure 4B).

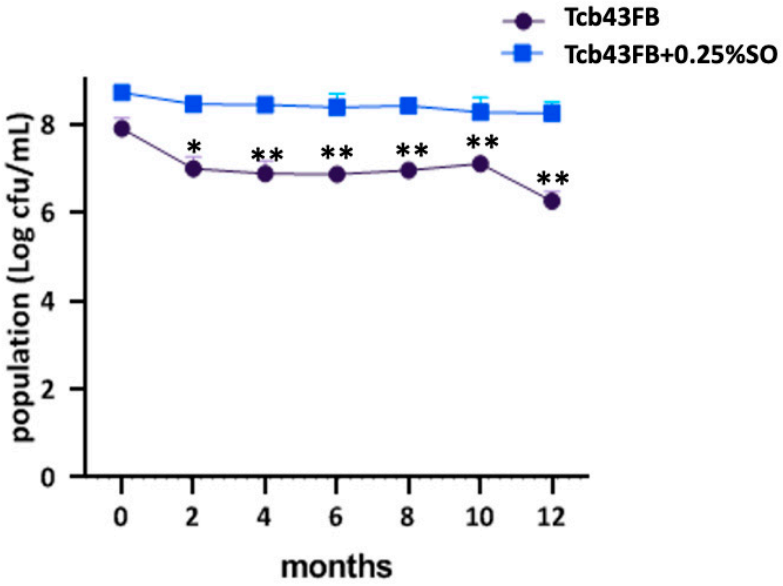

Figure 3. The shelf life analysis of *Bacillus velezensis* Tcb43 fermentation formula over 12 months. The shelf life of *B. velezensis* Tcb43 fermentation formula in a span of 12 months. Two formulas were tested: the basic fermentation broth formula (Tcb43FB) and the formula supplemented with 0.25% soybean oil (Tcb43FB + 0.25%SO). The endospore contents of Tcb43 in both fermentations were measured every two months and compared to the initial population. Statistical analysis was performed using Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$). Symbols represent the mean of 3 technical replicates, while error bars represent standard errors of the mean.

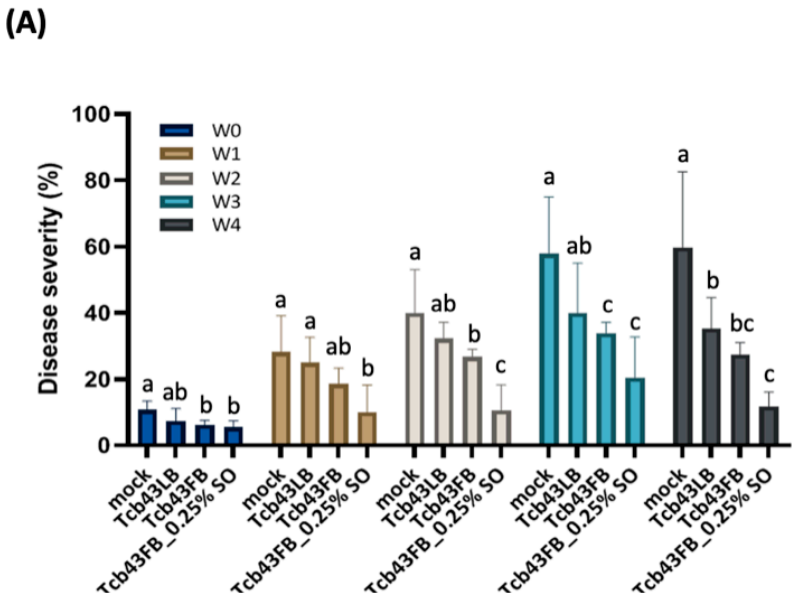

Figure 4. *Cont.*

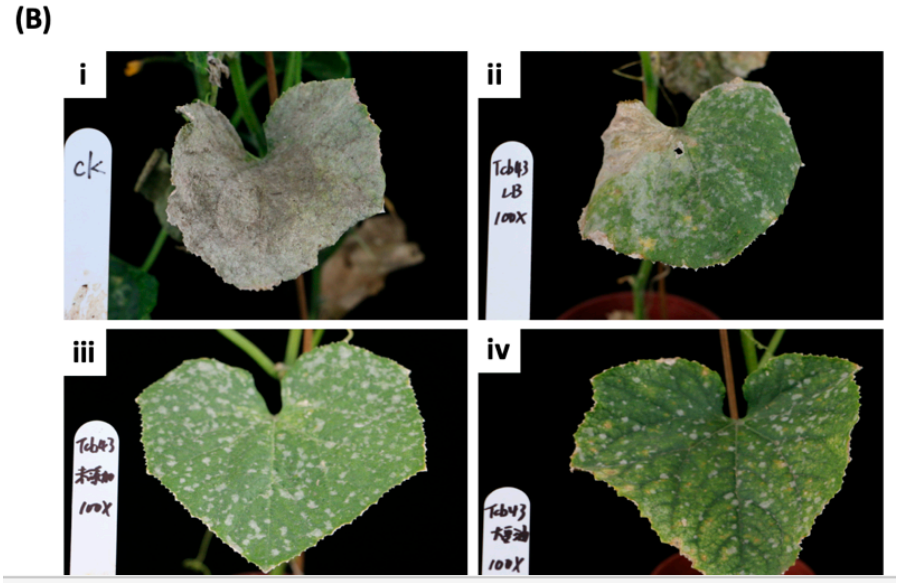

Figure 4. Biocontrol efficacy of *Bacillus velezensis* Tcb43 fermentation formula in a greenhouse assay. (**A**) Disease severity of powdery mildew in potted cucumber plant treated with Tcb43 fermented in LB (Tcb43LB), Tcb43 fermentation broth (Tcb43FB), and Tcb43 fermentation broth supplemented with 0.25% soybean oil (Tcb43FBSO). Different letters (a, b, or c) indicate significant differences based on Tukey's HSD test ($p < 0.05$). (**B**) Visual comparison of cucumber plants treated with different Tcb43 fermentation broth formulations after 4 weeks. Treatments: mock (**i**); 100-fold dilution of Tcb43LB (**ii**); Tcb43FB (**iii**); and Tcb43FBSO (**iv**).

3.6. Large-Scale Greenhouse Trials

The efficacy of Tcb43 fermentation broth mixed with soybean oil (Tcb43FBSO) in controlling powdery mildew was evaluated in large-scale greenhouses trials for cucumber crops during 2020 and 2023. Figure 5 shows three repeated large-scale greenhouse trials, showing similar trends. The data for the three trials were combined for statistical analysis. In the mock group, the disease severity increased gradually from 1.9 and 3.1% at week 0 (W0) and in the first week (W1) after administration, then to 16.6% in the second week (W2), 23.3% in the third week (W3), 59.3% in the fourth week (W4), and 84.5% in the fifth week (W5) (Figure 5A). In contrast, the disease severities of cucumber plants treated with 200-, 400- or 600-fold dilutions (200×, 400×, or 600×) of Tcb43FBSO fermentation broth were highest on W2 (4–7.3%), W3 (5.4–11.4%) and W4 (13–32.4%), and W5 (19.5–54.7%), showing that different dilutions of Tcb43FBSO did not significantly influence the disease severity on W2–W5. The control rates of these treatments ranged from 58.2% to 76.5% on W2, 49.3% to 77.1% on W3, 48.9% to 78.0% on W4, and 37.3% to 76.6% on W5, compared to the mock group.

The overall disease progression was evaluated using the area under the disease progress curve (AUDPC). The AUDPC values for the mock group ranged from 770.0 to 1449.3%-days, while the values for the 200×, 400×, and 600× dilutions of Tcb43 were 151.2–336.0, 292.5–767.1, and 383.2–878.6%-days, respectively. The AUDPC value of the Tcb43FBSO-treated group was significantly lower than that of the mock group. Specifically, the 200× treatment group showed a reduction in overall disease progression by 76.8–80.4% compared to the mock group. In Figure 5B, visual observations of the cucumber leaves revealed that the mock group exhibited white powdery mildew fungi covering the leaves, while the 200×-Tcb43FBSO-treated group showed very healthy leaves, indicating effective disease control.

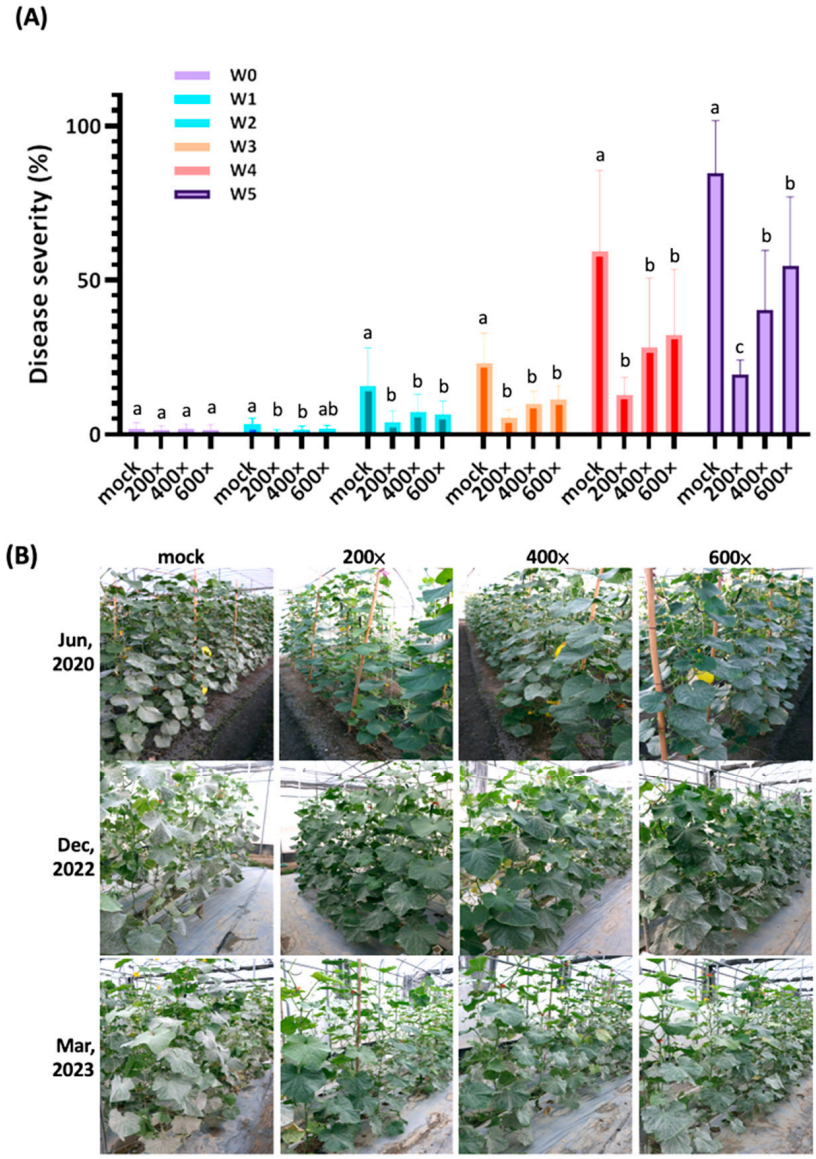

Figure 5. Effectiveness of *Bacillus velezensis* strain Tcb43 in controlling powdery mildew disease in three large-scale greenhouse cucumber trials. The greenhouse trials were conducted in Taichung, Taiwan from 2020 to 2023, where powdery mildew disease caused by *Podosphaera xanthii* was prevalent. (**A**) Disease severity progression of powdery mildew on Chugi variety cucumber plants treated with Tcb43 containing 0.25% soybean oil fermentation broth (Tcb43FBSO) at 200-, 400- and 600-fold dilutions (200×, 400×, and 600×), or water (mock control) over a five-week period. Disease severity was determined on week 0 (W0), 1 (W1), 2 (W2), 3 (W3), 4 (W4) (the last treatment of Tcb43 FBSO), and week 5 (W5) in each trial. Means within the columns followed by different letters are significantly different according to Tukey's HSD analysis ($p < 0.05$, n = 10). (**B**) Images of cucumber plants after being treated with water (mock) or Tcb43FBSO at 200×, 400×, and 600× dilution treatments on W5 showed the effectiveness of Tcb43 in controlling powdery mildew disease in June 2022, December 2022, and March 2023. Columns represent mean of 4 technical replicates and error bars represent standard error of the mean.

4. Discussion

Powdery mildew diseases commonly exhibited symptoms on leaves, young branches, and occasionally on fruits of various agricultural and ornamental plants [30]. The symptoms are characterized by the presence of powdery-looking patches or a diffusive layer of white, yellow, brown, or grayish mycelia and conidiophores on the plant surfaces of the

plants. This disease is a serious threat to the production of various crops, including those belonging to the Cucurbitaceae family [30,31]. Currently, some biofungicides have been registered and recommended for the control of cucumber powdery mildew disease in many countries [9,32]. However, there is limited information on the application of biopesticides to control powdery mildew in cucumber plants in Taiwan.

In our previous study, the Tcb43 strain isolated from an organic farm showed excellent antagonistic ability against *Colletotrichum lagenarium*, *C. capsici*, *C. gloeosporioides*, *Corynespora cassiicola*, *Stagonosporopsis cucurbitacearum*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Monosporascus cannonballus*. Furthermore, the application of the Tcb43 fermentation broth can significantly inhibit the germination of conidia of cucumber powdery mildew fungus [21]. Building upon these findings, we aimed to improve the fermentation formula to enhance the antagonistic activity of Tcb43 as a bioagent and suppress powdery mildew disease in large-scale greenhouse trials. We established the optimum carbon-to-nitrogen ratio to be 3% (*v*/*v*) in the fermentation nutrients (Figure 1) and found that the addition of soybean oil at 0.25% (*v*/*v*) increased the stability of the Tcb43 population (Figure 2) with a shelf life of one-year at room temperature (Figure 3). Tcb43, when applied to the potted cucumber plants in both the greenhouse and large-scale greenhouse trials, could effectively reduce the incidence of cucumber powdery mildew (Figures 4 and 5). Additionally, Tcb43 was tolerant to high alkalinity, acidity, salinity, and temperature and displayed resistance to most tested fungicides. These features demonstrated the potentiality of Tcb43 being used as a bioagent for future applications.

To gain insight into the gene function characteristics of Tcb43, the genome of Tcb43 was sequenced. Using the ANI value [24] for species identification, the genome sequence of Tcb43 (accession number JAQNDE000000000) exhibited higher similarity to the *B. velezensis* FZB42 strain. Consequently, Tcb43, previously identified as *B. amyloliquefaciens* based on sequences of *16S rRNA* and *gyrB* genes [21], was reclassified to *B. velezensis*. In addition, the analysis conducted using the anti-SMASH website accessed on 9 May 2022 (https://antismash.secondarymetabolites.org/#!/start) [33] revealed that the gene clusters of Tcb43 shared 100% similarity with the *B. velezensis* FZB42 strain, including macrolactin H (polyketide), fengycin (nonribosomal peptide synthetases, NRPs), bacillaene (polyketide + NRPs), difficidin (polyketide + NRPs), bacilysin, bacillibactin (NRPs), and other predicted metabolites such as terpenes, T3PKS, and various types of NRPs. In the previous study, Tcb43 produced several metabolites, like fengycin, surfactin, and Iturin [21]. These *Bacillus* lipopeptides, such as surfactins, iturins, fengycins, and agrastatin/plipastatins, were effective in inhibiting a wide range of phytopathogens via their antagonistic activity [9,34,35].

Our results indicated there were no significant differences in the growth of Tcb43 in either the Tcb43FB or Tcb43FBSO broths; however, the Tcb43FBSO broth did extend the shelf life (Figure 2), provided better antagonistic ability, and effectively controlled disease. Most vegetable oils, especially, soybean oil, are widely reported to enhance biosurfactant production in various microorganisms compared to other carbon sources like glycerol, glucose, or other hydrocarbons [36]. Soybean oil also contains a lot of fatty acids, such as palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, with linolenic acid specifically reported to enhance biosurfactant production [36]. Lipopeptide biosurfactants, including surfactin, fengycin, and polymyxins, exhibit antifungal activity due to their lipid tail length of 14–16 carbon atoms. This leads to various effects, including membrane disruption, increased permeability, the leakage of metabolites, the alteration of membrane structure and protein conformation, and ultimately, membrane dysfunction, cell lysis, and cell death. These mechanisms contribute to the effectiveness of lipopeptides in combating fungal pathogens [37,38]. In another case, the antagonistic strain *B. subtilis* UMAF6639 was specifically selected for controlling the cucurbit powdery mildew fungus *Podosphaera fusca*. The UMAF6639 strain activates jasmonate- (JA) and salicylic acid (SA)-dependent defense responses in melon to provide additional protection against powdery mildew [9,39], including the production of reactive oxygen species and reinforcement of the cell wall [39]. The surfactin lipopeptide produced by UMAF6639 plays a crucial role in stimulating

the immune response. Overall, UMAF6639 confers protection against cucurbit powdery mildew through the activation of these defense mechanisms [39]. Additionally, the plant-growth-promoting properties of the biosurfactant-producing bacterium, *B. tequilensis* LK5.4, was evaluated, and the treated plants showed increased moisture content due to higher bioadsorption [40].

Therefore, the Tcb43 strain, which is capable of producing various biosurfactants, has the potential to prevent and control cucumber powdery mildew disease through its antifungal activity. However, whether Tcb43 performs other functions such as inducing systemic resistance in plants, competition for nutrients and space with pathogens, and having plant-growth-promoting activity or producing other antagonistic substances [25,41,42] warrants further investigation.

5. Conclusions

Powdery mildew is caused by the biotrophic pathogen *Podosphaera xanthii*. We identified theTcb43 strain of *Bacillus velezensis* and demonstrated its effectiveness in controlling powdery mildew disease in cucumber in large-scale greenhouse trials. We found that the addition of 0.25% soybean oil during fermentation could enhance the sporulation of Tcb43 and greatly extend the shelf life for storage. Large-scale greenhouse trials on cucumber plants have also demonstrated that applying at least a 200-fold dilution of Tcb43 could achieve more than a 37.3–76.6% reduction in powdery mildew disease in cucumber. These results indicate that the development of Tcb43 as an effective bioagent for controlling cucumber powdery mildew disease is warranted.

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