

## Article

# Selection, Formulation, and Field Evaluation of *Bacillus amyloliquefaciens* PMB01 for Its Application to Manage Tomato Bacterial Wilt Disease

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**Abstract:** Bacterial wilt caused by the soil-borne pathogen *Ralstonia solanacearum* is one of the most devastating diseases in solanaceous plants. No agrochemicals are available to manage bacterial wilt effectively. A *Bacillus amyloliquefaciens* strain designated PMB01 was recovered from the cabbage rhizosphere and was found to be capable of inhibiting the growth of *R. solanacearum*. The PMB01 strain was highly resistant to extreme pH, heat, high salt salinity, and various fungicides. In contrast, PMB01 was sensitive to copper-based compounds, streptomycin, and tetracycline. The efficacy of the PMB01 strain in suppressing *R. solanacearum* and bacterial wilt in tomatoes was significantly improved when the culture medium was supplemented with 1% (*w/v*) soybean meal. PMB01 was in a 500-liter tank for the pilot production, and the resultant broth could effectively reduce the severity of tomato bacterial wilt in greenhouse trials. The PMB01 fermentation broth was mixed with 10% corn starch and 30% maltodextrin to make a wettable powder (WP). PMB01 could survive in the wettable powder for more than two years without losing its antagonistic activity. In ten field trials, tomato plants treated with 50, 100, or 200-fold dilutions of PMB01 WP reduced bacterial wilt severity by more than 67% compared to the mock (water control) treatment. This work revealed that the effectiveness of the rhizobacterium PMB01 to antagonize *R. solanacearum* was greatly improved when the culture medium was supplemented with 1% (*w/v*) soybean meal, indicating that PMB01 is an ideal bio-agent candidate. A durable format suitable for storage was also developed. Similar concepts may be applied to other bio-agent candidates to improve their effectiveness in disease management.

**Keywords:** *Bacillus amyloliquefaciens*; bacterial wilt disease; biocontrol; fermentation; *Ralstonia solanacearum*



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## 1. Introduction

Bacterial wilt disease caused by soil-borne *Ralstonia solanacearum* is one of the most devastating bacterial diseases in the tropics, subtropics, and some temperate areas with humid and hot summers, with phylotype IIB-1 strains that are acclimatized to even lower temperatures and have caused significant outbreaks in temperate areas in recent years [1,2]. *R. solanacearum* affects more than 200 plant species, including many solanaceous plants [3,4]. The yield loss of tomato varies up to 91%, and the losses vary from about 10% to 30% in tobacco and from 33% to 90% in potato [5]. Tomato plants infected by *R. solanacearum* often show stunting, leaf wilting and chlorosis, vascular discoloration, and death [6]. Bacteria constantly oozed from the cut end of diseased stems when placed in water. *R. solanacearum* can be transmitted in various ways, such as root contact, irrigation water, machinery, or insects [3,4,7,8].

*R. solanacearum* can survive in soils for more than ten years without host plants [9]. The bacterial wilt caused by *R. solanacearum* is difficult to control as the pathogen grows

endophytically and survives well in soil [10]. Until now, there are no pesticides that could effectively reduce the occurrence of this disease. To manage bacterial wilt disease, various methods, including crop rotation, field sanitation, using the resistant variety, and soil disinfection must be implemented [11–13]. Current trends in plant disease management reveal that more attention has been paid to applying biological control agents to control various plant diseases [14–16].

Many microorganisms with the capability to suppress the bacterial wilt of solanaceous plants have been reported. These include fluorescent pseudomonads (*Pseudomonas putida* and *P. fluorescens*), actinomycetes (*Streptomyces* spp.), *Pantoea* spp., and *Bacillus* spp. [17–21]. Among them, the spore-forming *Bacillus* spp. are not only a plant growth-promoting bacteria (PGPR) that provide benefits through the activity of nitrogen fixation, induction of phytohormones, or siderophore production for iron sequestration [22–24], but also are able to control plant diseases due to the production of lipopeptide antibiotics such as surfactin and iturin, which can inhibit the growth of many phytopathogens [25–28]. *Bacillus* spp. have often been used to develop biopesticide products [11,29] because lipopeptide compounds are stable, have broad-spectrum antimicrobial activity, and can last for an extended period without losing antagonistic activities [19,30]. Many commercially available bioproducts are sold in a liquid form or powder [31,32]. Despite copious studies on biocontrol, there is no commercially available biopesticide to control the bacterial wilt disease of tomatoes [33]. Because of the ineffectiveness of pesticides on *R. solanacearum* and the severity of bacterial wilt disease worldwide, there is an urgent need to develop effective biopesticides for controlling the bacterial wilt of tomatoes. The products must be effective, easily mass-produced, stored, and delivered. The biopesticides must be tested in field conditions to ensure their effectiveness and durability in controlling bacterial wilt disease.

In the present study, we identified a heat-tolerant *B. amyloliquefaciens* strain (designated PMB01) isolated from a cabbage field and demonstrated its capability of suppressing bacterial wilt disease in greenhouse and field trials. We have also found that adding soybean meal into the medium during fermentation could significantly enhance spore formation and the antagonistic activity of *B. amyloliquefaciens*. The *B. amyloliquefaciens* PMB01 strain was formulated as a wettable powder and tested for its ability to inhibit *R. solanacearum* for two years. Greenhouse trials revealed that PMB01 could effectively suppress the bacterial wilt disease of tomatoes. Ten-year field trials also showed that PMB01 WP could reduce the disease severities by at least 67% in different tomato farms with naturally occurring bacterial wilt disease. Our results demonstrated that PMB01 is a promising candidate for future development as a biocontrol agent to effectively manage bacterial wilt diseases.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Media

The AL03 strain of *Ralstonia solanacearum* (*Rs*) was isolated from a diseased tomato plant (cv. Yu Nu) in A-Lian District (Kaohsiung, Taiwan) and was used for bioassays. *Rs*MN01 and *Rs*RW01 strains were isolated from diseased cherry (cv. Golden Sweet 4111) and beef (cv. TMB-688) tomatoes, respectively, and used in antagonistic tests. Fifteen rhizobacterial strains showing antagonistic activity against the *Rs* strains were isolated from different vegetable rhizospheres in Pingtung County and Kaohsiung County (Taiwan). All bacterial strains were stored in 20% (*v/v*) glycerol at  $-80\text{ }^{\circ}\text{C}$ . Bacteria were routinely cultured at  $30\text{ }^{\circ}\text{C}$  on Luria-Bertani (LB) medium for rhizobacteria and triphenyl tetrazolium chloride medium (TTC) for the *Rs* strains. The TTC medium contained 1% Bacto peptone, 0.1% casein hydrolysate (Sigma-Aldrich, Burlington, MA, USA), 0.5% glucose, 0.005% 2,3,5-triphenyl-2H-tetrazolium chloride (Thermo Fisher Scientific, Waltham, MA, USA), and 1.5% Bacto agar (pH 7.2). All ingredients were prepared based on weight per volume (*w/v*) unless otherwise indicated.

## 2.2. Isolation of Rhizobacteria and In-Vitro Antagonistic Assays

Soils were collected from rhizospheres in vegetable farms, suspended in sterile water, and heated at 70 °C for 30 min. After cooling down to room temperature, 100- $\mu$ L suspensions were plated onto Difco Nutrient Agar (0.3% beef extract, 0.5% peptone, and 1.5% agar, pH 7.0). After incubation at 30 °C for 24–48 h, bacteria were selected for antagonistic activity assays. The radius of the inhibitory zone against RsAL03, RsMN01, and RsRW01 was measured. Antagonistic assays were performed to characterize the inhibitory activity by culturing rhizobacteria at the center of the TTC medium at 30 °C for 24 h, followed by evenly plating the suspension of pathogenic strains RsAL03, RsMN01, or RsRW01 at the concentration of OD<sub>600</sub> of 0.3 (approximate  $5 \times 10^8$  CFU/mL) onto the TTC medium and incubated at 30 °C for 24 h.

## 2.3. Identification and Characterization of Antagonistic Rhizobacterium

### 2.3.1. Molecular Identification

The identity of PMB01 was determined by sequence analysis of the highly conserved region of the 16S ribosomal RNA gene (16S *rRNA*) and the DNA gyrase subunit A gene (*gyrA*). Genomic DNA (gDNA) of PMB01 was extracted using a DNA Mini kit (Zymo-BIOMICS, Irvine, CA, USA). The 16S *rRNA* fragment was amplified by PCR with the primers 8F (16S *rRNA* forward, 5'-AGAGTTTGATCCTGGCTCAG-3') [34] and 1520R (16S *rRNA* reverse, 5'-AAGGAGGTGATCCAGCCGCA-3') [35]. The *gyrA* fragment was amplified with the primers p-*gyrA*-F (*gyrA* forward, 5'-CAGTCAGGAAATGCGTACGTCCTT-3') and p-*gyrA*-R (*gyrA* reverse, 5'-CAAGGTAATGCTCCAGGCATTGCT-3') [36]. The PCR amplification was performed with a T3000 Thermocycler (Biometra GmbH, Göttingen, Germany) using the following conditions: a denaturing step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. DNA fragments were directly sequenced using an Applied Biosystem 3730 DNA analyzer (Waltham, MA, USA) at Genomics BioSci & Tech Co., (New Taipei City, Taiwan). Sequence similarity searching was conducted using BLAST in the NCBI GenBank.

### 2.3.2. BIOLOG GEN III System

One of the tested rhizobacteria, PMB01, was selected for Biolog analysis. The bacterial strain was streaked on Biolog Universal Growth (5.7% BIOLOG BUG™ agar, BIOLOG, Hayward, CA, USA), incubated at 30 °C for 4–16 h for single colonies, and tested for physiological metabolisms following the manufacturer's instructions. Briefly, the suspension of PMB01 was cultured on 96-well Biolog GEN III MicroPlates and assayed with the BIOLOG GEN III MicroStation System in three intervals of 6–8 h, 16–24 h, and 24–48 h. The data were compared to the BIOLOG's Microbial Identification Systems database using MicroLog 3 Software to determine the possible identity of PMB01.

### 2.3.3. Assays for Sensitivity to Stress, Fungicides, Copper Agents, and Antibiotics

Salt tolerance was conducted by growing PMB01 on LB amended with 7%, 8%, or 10% NaCl. To test heat tolerance, PMB01 suspensions were boiled at 100 °C for 30 min and spread on LB. To test pH tolerance, PMB01 was cultured on LB with the adjustment of pH ranging from 3 to 10. To test sensitivity to fungicides, copper agents, and antibiotics, PMB01 was cultured on LB amended with one of the test chemicals. The chemicals used in this study included: 0.05% (*w/w*) Iprodione wettable powder (WP) (Great Victory Chemical Industry Co., Yunlin, Taiwan), 0.0118% (*w/v*); Pyraclostrobin emulsifiable concentrate (BASF Taiwan, Taipei, Taiwan), 0.025% (*w/w*); Benomyl WP (Fulon Chemical Industrial Co., Taoyuan, Taiwan), 0.035% (*w/w*); Metalaxyl WP (Jianon Enterprise Co., Hsinchu, Taiwan), 0.04% (*w/w*); Iminoctadine tris (also known as albesilate) WP (Yih Fong Chemical Corp., Taipei, Taiwan), 0.05% (*w/w*); Flutolanil WP (Taiwan Nihon NohyaKu Co., Taipei, Taiwan), 0.034% (*w/w*); Tribasic copper sulfate SC (Taiwan Nissan Chemical Industries Corp., Taipei, Taiwan), 0.0385% (*w/w*); Copper hydroxide WP (Sinon Corporation, Taichung, Taiwan),

0.0125% (v/v); streptomycin soluble concentrate (Lih-Nung Chemical Co. Yunlin, Taiwan), and streptomycin + tetracycline water soluble powder, 0.01% (w/w) (All Taiwan Pesticides Co., Taipei, Taiwan). All pesticides were prepared according to the manufacturer's recommendation. All assays for chemical compound sensitivity evaluation were performed by growing PMB01 at 30 °C for 24 h.

#### 2.4. Preparation of Fermentation Broth for Inhibitory Assays

Fermentation was carried out in three scales: small, bench, and pilot, with broth volumes of 250 mL, 10 L, and 500 L, respectively. PMB01 was cultured in a 250-mL LB at 30 °C for 24 h and transferred to a 10-L fermentation medium (pH 7.0) containing 3% molasses, 1% yeast extract, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 mM MnSO<sub>4</sub>, and different concentrations (0, 0.5%, 1%, and 1.5%) of soybean meal (Central Union Oil Corp, Taiwan). Fermentation was carried out in a Winpact Evo Fermentation System FS-07 (15.75'' × 23.62'' × 29.14'', Major Science, Taoyuan, Taiwan) equipped with a stirrer set at 200 rpm and ventilation set at 5.5 LPM (liter per min). After being cultured in the fermentation system at 30 °C for 24 h, 50 µL suspensions were plated on TTC and tested for antagonistic ability against RsAL03. The pilot production was carried out in the fermentation system containing a 500-L fermentation medium amended with 1% soybean meal with a constant stirring set at 80 rpm and normal aeration volume (100 LPM) at 30 °C for 96 h. The population of PMB01 during the pilot fermentation was determined by viable cell counts at 24-h intervals. The number of spores was determined by heating the broth at 70 °C for 30 min and spreading it on the medium plate as described [37]. The sporulation ratio of PMB01 was calculated as the number of spores divided by the number of viable cells. After fermentation, the broth was mixed with 10% (v/v) corn starch and 30% (v/v) maltodextrin to make a wettable powder (Jianon Enterprise Co., Hsinchu, Taiwan) and stored at room temperature (25–28 °C). The viability of PMB01 was examined every three months for two years.

#### 2.5. Greenhouse Trials for Managing Tomato Bacterial Wilt

Tomato seeds cv. Known-You 301 (Known-You Seed Co., Kaohsiung, Taiwan) were planted in peat moss (BVB no. 4, Bas Van Buuren, Maasland, New Zealand) in an 8 × 16 seedling tray. Seedlings were grown in a glass greenhouse (daily cycle: 18 h light at 28 °C, 8 h dark at 25 °C) for 2–3 weeks and transplanted to 5-inch pots. *R. solanacearum* RsAL03 was cultured in a 523 medium (1% sucrose, 0.8% casein enzymatic hydrolysate, 0.4% yeast extract, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.03% MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7) [38] for 2 days and suspended in distilled water at OD<sub>600</sub> of 0.3 for inoculation. Tomato seedlings were inoculated with the prepared RsAL03 suspensions by soil drenching (50 mL/pot), followed by drenching with 50 mL diluted pilot fermentation broth containing 10<sup>7</sup>–10<sup>9</sup> CFU/mL of PMB01. In the greenhouse experiments, the rhizosphere of tomato seedlings was drenched with 50 mL distilled water (mock control), the fermentation medium only (blank control), and the PMB01 fermentation broth. Each treatment contained 15 seedlings, and experiments were repeated three times. Treatments were performed every 7 days, and the disease severity of bacterial wilt was determined weekly [39]. Disease rating was conducted using a 0–4 scale: 0, no symptoms; 1, one leaf or two leaves wilted; 2, three or four leaves wilted; 3, five or six leaves wilted; and 4, all leaves wilted or plant death. The following formula was used to calculate the disease severity: Disease severity (%) = [Σ (scale value × number of infected plants at each scale value)/total number of plants × 4] × 100.

#### 2.6. Field Trials

From 2014 to 2020, ten field trials were conducted in 10 naturally infested tomato farms in Ren-Wu, Luzhu, Qishan, A-Lian, and Mei-Nong Districts in Kaohsiung City and in Nanzhou Township in Pingtung City to evaluate the efficacy of PMB01 for controlling bacterial wilt disease. Bacterial wilt occurred regularly in these farms. Three tomato cultivars, such as beef tomato 'TMB-688', cherry tomato 'Yu Nu', and cherry tomato

'Golden Sweet 4111, were planted in these farms (Supplemental Table S1). Considering that the applied PMB01 would remain in the soil, the experiment was performed only once on each farm. Tomato plants flower 30–40 days after transplanting and can be harvested 70–80 days after transplanting in Southern Taiwan. Since bacterial wilt usually began at the flowering stage, the experiment was carried out from 40 to 75 days after transplanting.

PMB01 ( $1 \times 10^9$  CFU/mL) WP was dissolved, diluted in water, and drenched into soils (200 mL per plant) manually. Tomato plants were treated with 200X, 100X, or 50X dilution of the PMB01 WP or water (mock). The experiments were conducted following a randomized complete block design [40], with four replicates per treatment per farm. On each farm, there were four blocks each containing four plots, and the four treatments were assigned randomly to the four plots. The spacing between blocks and between plots was 240–250 cm. In each plot, a total of 60 plants were planted in two rows (30 plants per row), with 60–70 cm between rows and 40–50 cm between plants. PMB01 WP was applied weekly for five weeks continuously. Before each application, the severity of bacterial wilt disease was scored. The first disease rating was performed 40 days after transplanting, immediately before the first application of PMB01 (day 0). The final disease rating was conducted 7 days after the last application of PMB01 (day 35). Disease rating was conducted using a 0–6 scale as follows: 0, no disease; 1, 1% to 15% leaves wilted; 2, 16% to 30% leaves wilted; 3, 31% to 45% leaves wilted; 4, 46% to 60% leaves wilted; 5, 61% to 75% leaves wilted; and 6, more than 76% leaves wilted (modified from the method of Roberts et al.) [41]. Disease rating was conducted on 30 randomly selected plants per plot (randomization was conducted using Microsoft Excel 2013). The disease severity of each plot was calculated as: Disease severity (%) =  $[\sum (\text{scale value} \times \text{number of infected plants at each scale value}) / \text{total number of plants} \times 6] \times 100$ . 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  $y_i$  = disease severity at time  $i$ ,  $t_{i+1} - t_i$  = day interval between two ratings,  $n$  = number of ratings [42].

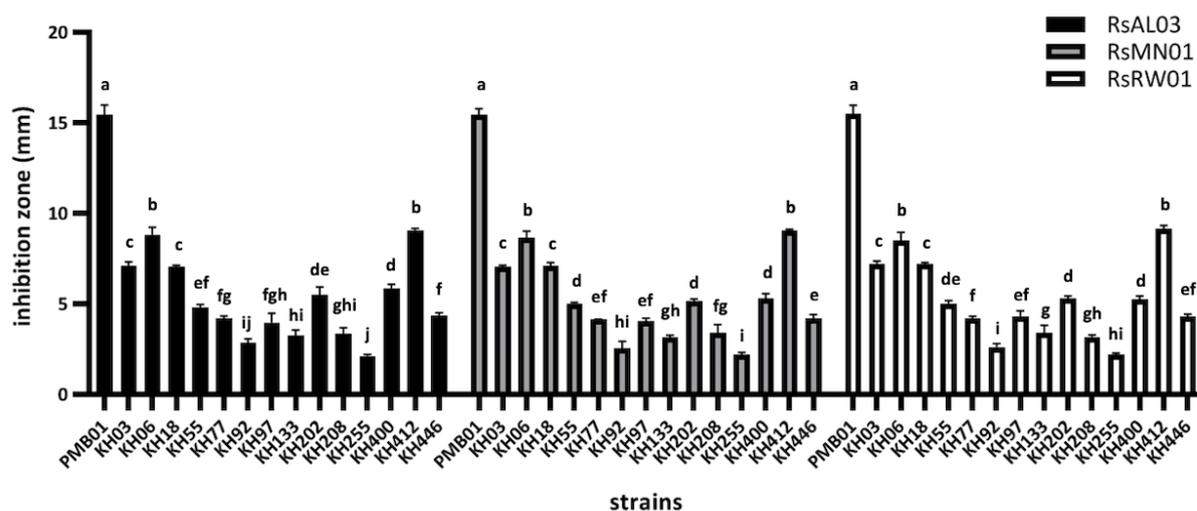
### 2.7. Statistical Analysis

Data from each experiment were tested for homogeneity of variance and analyzed for variance using the SPSS statistics software Version 20 (IBM Corp., Armonk, NY, USA). Because the environmental conditions (farm site, cultivar, and month-year) of the 10 field trials were different, data from each field trial were analyzed separately. Analysis of variance (ANOVA) was used to test the effect of different treatments in all assays. Differences between treatments were determined by Tukey's HSD analysis or Student's  $t$ -test ( $p < 0.05$ ).

## 3. Results

### 3.1. Isolation of Rhizobacteria and In-Vitro Antagonistic Activity Assays

After soil suspensions were treated at 70 °C for 30 min, 15 bacteria were recovered. Antagonistic activity assayed on TTC plates revealed that most heat-tolerant rhizobacterial strains showed antagonistic activity against three test *R*s strains. Eight rhizobacterial strains showed inhibitory radii ranging from 0 to 5 mm, and six strains showed inhibitory radii from 5 to 10 mm. A strain designated PMB01 showed an inhibitory radius greater than 15 mm (Figure 1).



**Figure 1.** Antagonistic effects of 15 *Bacillus amyloliquefaciens* strains against three *Ralstonia solanacearum* strains evaluated by forming an inhibition zone (mm) on a triphenyl tetrazolium chloride medium (TTC). Means within the columns followed by different letters are significantly different according to Tukey's HSD test ( $p < 0.05$ ).

### 3.2. Identification of PMB01

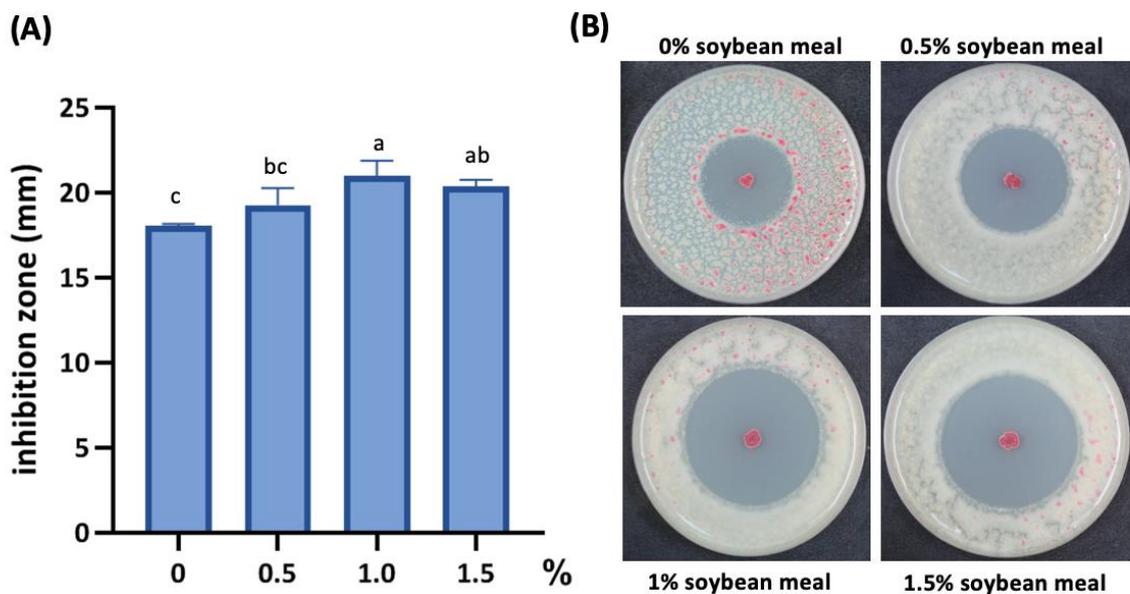
BIOLOG GEN III system assays revealed that PMB01 utilizes various nutrient sources, including D-glucose, sucrose, D-mannose, D-fructose, D-lactose, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, and L-serine. The analyses using a Biolog GEN III MicroStation system and the comparison with Biolog's database, PMB01 showed similarity indexes (indicated in parentheses) to *Bacillus amyloliquefaciens* (0.636), *B. subtilis* (0.011), *B. pumilus* (0.003), and *B. megaterium* (0.001). The identity of PMB01 was further confirmed by analyzing sequences of two conserved genes. PMB01 16S rRNA sequence (accession number: OP320919) showed 99.38% nucleotide identity with that of the *B. amyloliquefaciens* GBSW11 strain (GU568203.1). PMB01 *gyrA* gene sequence (2382 bp, accession number: OP328915) showed 100% identity with that of the *B. amyloliquefaciens* LM2303 strain (CP018152.1).

### 3.3. PMB01 Is Highly Resistant to Stress and Fungicides

Physiological and biochemical tests revealed that PMB01 grows at a pH ranging from 3 to 10 and salt concentrations ranging from 7% to 10%. More than 98% of PMB01 cells were recovered after being heated at 100 °C for 30 min. The growth of PMB01 was not affected by Iprodione, Pyraclostrobin, Benomyl, Metalaxyl, Iminoctadine tris (albesilate), and flutolanil at the test concentration. PMB01 was insensitive to streptomycin (0.0125%). However, PMB01 was highly sensitive to copper-based compounds (such as tribasic copper sulfate or copper hydroxide) and streptomycin + tetracycline.

### 3.4. Soybean Meal Enhances the Inhibitory Activity of PMB01

To test the fermentation formula to obtain better antagonistic activity of PMB01, the antagonistic tests assayed on TTC plates amended with PMB01 fermentation broth revealed that adding soybean meal significantly enhanced the antagonistic activity of PMB01 (Figure 2A). Adding 1% soybean meal during PMB01 fermentation gave the highest inhibition rate of *Rs* (Figure 2B). For the fermentation process in a 1% soybean meal-containing medium in a 500-L tank, PMB01 reached above  $10^7$  CFU/mL 24 h after incubation (hai). More than 74.39% of PMB01 cells produced endospores 24 hai and reached 100% 72 hai (Table 1). The PMB01 population was over  $10^9$  CFU/mL 72 hai.



**Figure 2.** Antagonistic effects of *Bacillus amyloliquefaciens* PMB01 strain against the RsAL03 strain of *Ralstonia solanacearum* on the TTC medium amended with different concentrations (0%, 0.5%, 1%, and 1.5%) of soybean meal. (A) Quantitative measurement of the inhibition zone (mm). (B) Images of the inhibition zone caused by PMB01 on the lawn of *R. solanacearum*. Means within the columns followed by different letters are significantly different according to Tukey's HSD test ( $p < 0.05$ ).

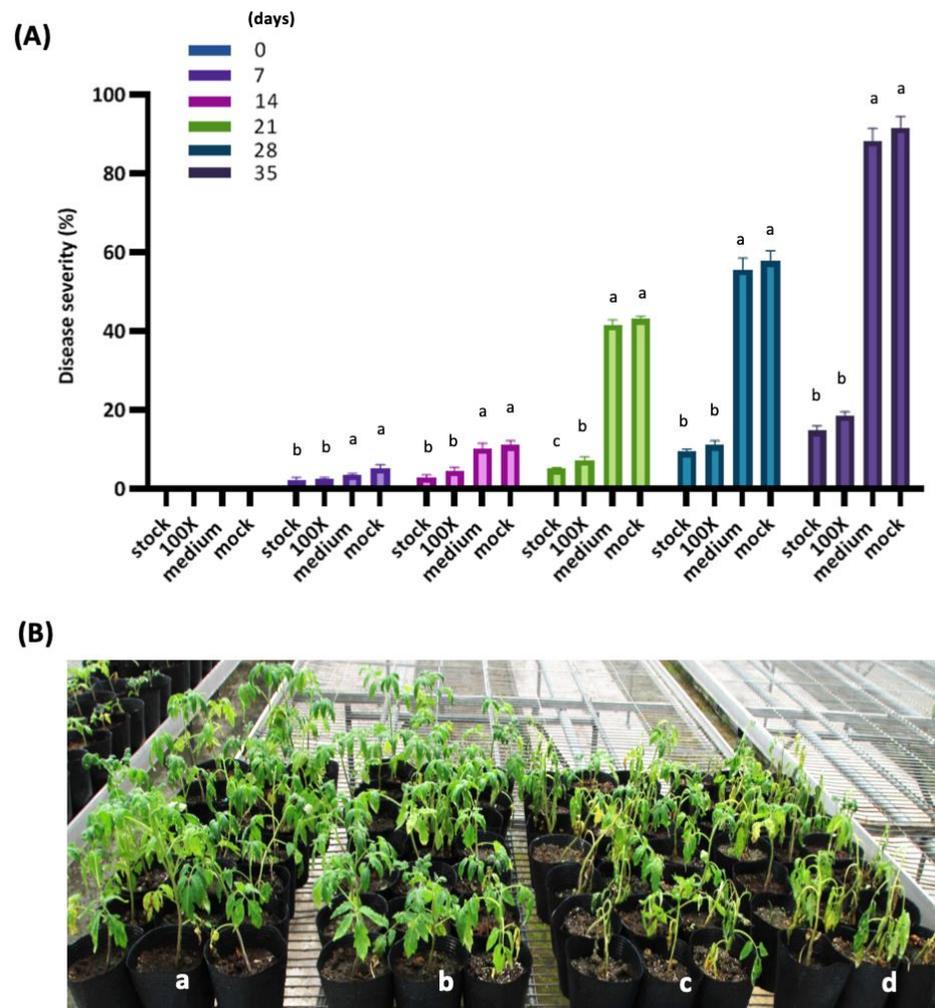
**Table 1.** Sporulation of *Bacillus amyloliquefaciens* PMB01 during fermentation in a 500-L tank.

Time (Hours)	Cells (CFU/mL)	Spores (CFU/mL)	Sporulation (%)
0	6.28 ± 0.04	0 ± 0 **	0
24	7.34 ± 0.07	5.46 ± 0.13 **	74.39
48	8.26 ± 0.07	8.07 ± 0.06 *	97.75
72	9.54 ± 0.02	9.54 ± 0.01	100
96	9.55 ± 0.03	9.55 ± 0.03	100

Means of cell and spore numbers are presented in Log values. The sporulation percentage was calculated based on the ratio of spores in living cells. The asterisks indicate a significant difference compared to the number of spore cells according to the Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

### 3.5. PMB01 Suppresses Bacterial Wilt Disease in Greenhouse Trials

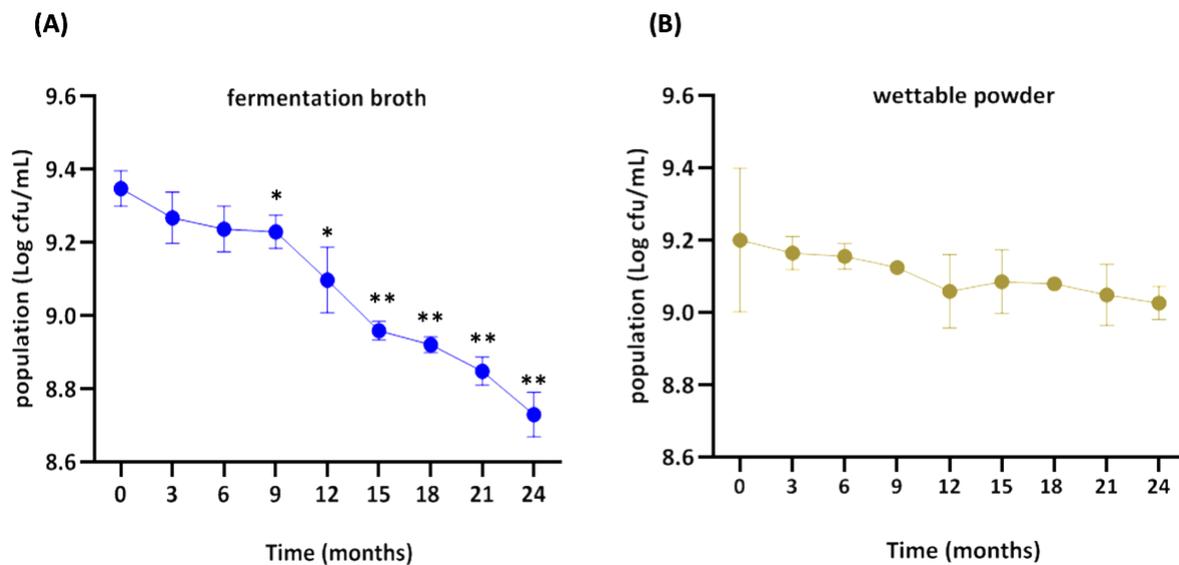
To verify the efficacy of PMB01 fermentation broth against bacterial wilt, a greenhouse experiment was performed to evaluate the biocontrol ability of PMB01. The results revealed that the disease severity of tomato bacterial wilt was reduced by adding the fermentation broth stock at 100-fold dilution (100X) compared with the water treatment or fermentation broth medium alone, 7 days post-application. The suppression of bacterial wilt disease was sustained until 35 days post-application. The disease severities of bacterial wilt were 14.7%, 18.6%, 88.3%, and 91.6% after the treatment with the stock of fermentation broth, 100X, medium alone, and water, respectively. However, there was no significant difference in the disease severity after being treated with the stock or 100X each week (Figure 3A). Most plants treated with PMB01 fermentation broth showed mild or no wilt symptoms (Figure 3B).



**Figure 3.** The effect of the *Bacillus amyloliquefaciens* PMB01 strain on controlling bacterial wilt of tomatoes in greenhouse conditions. **(A)** The disease severities of bacterial wilt were determined on days 0, 7, 14, 21, 28 (last treatment of PMB01), and 35. Means within the columns followed by different letters are significantly different according to Tukey's HSD analysis ( $p < 0.05$ ). **(B)** Images of tomato plants after being treated with or without PMB01 fermentation broth on day 35. Treatments: undiluted stock (a); 100X (100-fold dilution) (b); medium only (medium) (c); and water control (mock) (d).

### 3.6. PMB01 Formulated as a Wettable Powder Remains Active for 24 Months

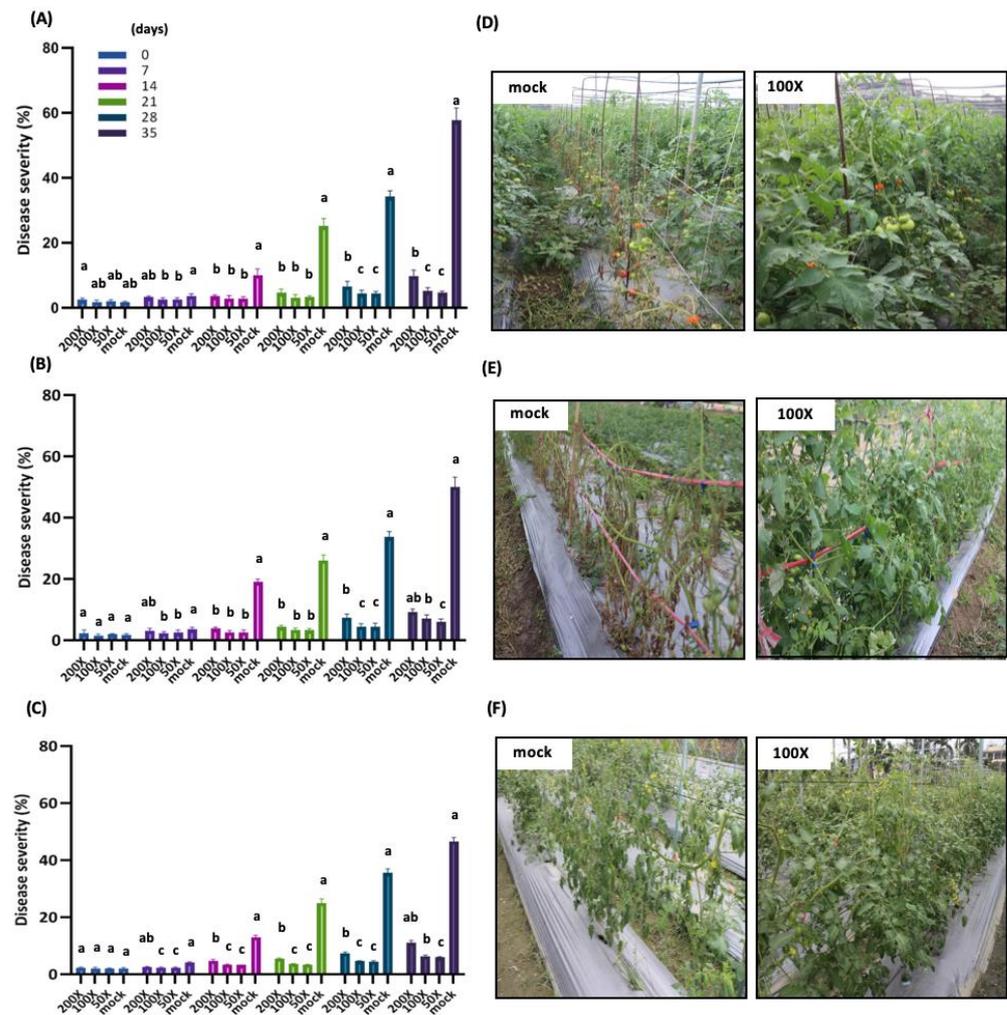
To improve its portability and convenient use in the field, PMB01 was formulated as wettable powders. The shelf life of the wettable powder and the fermentation broth of PMB01 were compared by bacterial vitality, showing that the efficacy of antagonistic activity of PMB01 fermentation broth against *Rs* declined considerably after 9-month storage at ambient temperature. The bacterial population decreased by 0.25 log CFU/mL (down from 9.35 log CFU/mL) after 12 months (Figure 4A). However, the bacterial population in the wettable powder decreased only by 0.14 log CFU/mL after 12 months. It remained viable with cell counts of 9.03 log CFU/mL after 24 months (Figure 4B). Statistical analyses revealed no significant difference in the population of PMB01 wettable powders between 0- and 24-month storage.



**Figure 4.** Survival of *Bacillus amyloliquefaciens* PMB01 strain in liquid fermentation broth (A) or wettable powder (B) in 24 months. The population of PMB01 was determined every three months, compared with the initial population, and analyzed statistically by Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

### 3.7. Applying PMB01 WP Suppresses Tomato Bacterial Wilt in the Field

The efficacy of PMB01 WP (50X, 100X, and 200X dilutions) in controlling tomato bacterial wilt was evaluated in 10 tomato farms between 2014 and 2020 (Supplemental Table S1). Three field trials showing the best control efficacies on different tomato cultivars are presented in Figure 5. These were beef tomato TMB-688 at Farm 1 in Ren-Wu District in Kaohsiung in 2014 (Figure 5A,D), cherry tomato cv. Yu Nu at Farm 5 in A-Lian District in Kaohsiung in 2015 (Figure 5B,E), and cherry tomato cv. Golden Sweet at Farm 8 in Mei-Nong District in Kaohsiung in 2016 (Figure 5C,F). Regardless of different cultivars and environmental conditions in the 10 field trials, similar trends of disease progression were observed for PMB01-treated and mock groups. For the mock group, disease severity started from 1.3–4.1% at 0 and 7 days post application (dpa), gradually increased to 6.0–19.0% at 14 dpa, 17.5–26.3% at 21 dpa, 27.3–36.6% at 28 dpa, and reached to as high as 39.8–57.7% at 35 dpa. Compared to the mock group, plants treated with PMB01 showed significantly lower levels of disease severity at 14 dpa (2.6–4.8%), 21 dpa (3.2–6.6%), 28 dpa (4.3–8.2%), and 35 dpa (4.7–13.0%). At 35 dpa, the disease severity was reduced by 68.0–91.8% with PMB01 treatment compared to the mock group. From 0 to 21 dpa, no significant differences were observed among 50X, 100X, and 200X dilutions of PMB01 WP (the only exception was 200X dilution at Farm 8). In some fields at 28 and 35 dpa, treatment of 200X dilution showed significantly higher levels of disease severity than 50X or 100X dilutions. The overall disease progress across the whole experimental period was represented by AUDPC. While the AUDPC of the mock groups ranged from 521.0–754.9 %-day, the AUDPC of 50X, 100X, and 200X dilutions of PMB01 were 113.7–165.1, 112.6–171.9, and 169.0–198.4 %-day, respectively. The AUDPC values of PMB01-treated groups were significantly lower than the mock groups; however, no significant difference was detected among different dilutions of PMB01. The overall disease progress was reduced by 67.3–84.8% with PMB01 treatment compared to the mock group.



**Figure 5.** Field trials evidencing the *Bacillus amyloliquefaciens* PMB01 strain's effectiveness in controlling bacterial wilt disease in three tomato farms. Bacterial wilt disease caused by *Ralstonia solanacearum* occurred regularly in these farms. (A) The disease severity of bacterial wilt on TMB-688 tomato variety planted in Ren-Wu District in 2014. (B) The disease severity of bacterial wilt on Yu Nu tomato variety grown in A-Lian District in 2015. (C) The disease severity of bacterial wilt on Golden Sweet tomato variety grown in Mei-Nong District in 2016. Tomato plants were treated with PMB01 wettable powder (WP) at 200X, 100X, 50X, or water (mock control) every week for five consecutive weeks. Disease severity was determined on days 0, 7, 14, 21, 28 (last treatment of PMB01), and 35 in each trial. Means within the columns followed by different letters are significantly different according to Tukey's HSD analysis ( $p < 0.05$ ). Images of tomato plants after being treated with water (mock) or PMB01 wettable powder at 100X on day 35, showing the effectiveness of PMB01 in controlling bacterial wilt in tomato farms located in Ren-Wu (D), A-Lian (E), and Mei-Nong (F) districts. In these three farms, plants treated with water only (mock) showed severe wilting, whereas plants treated with PMB01 WP (100X) were healthy in three locations.

#### 4. Discussion

The bacterial wilt of tomatoes is a severe disease worldwide, causing more than 90% yield loss in some regions. The disease severity is determined by various factors, including host cultivars, climate, soil type, cropping system, and virulence of *R. solanacearum* strains [33]. *R. solanacearum* invades its hosts from roots and spreads along the vascular bundle, which could rapidly lead to plant wilt [2,7]. Currently, no chemical agent can effectively control the occurrence of bacterial wilt disease [2,3]. Recommendations for controlling bacterial wilt include crop rotation, field sanitation, soil fumigation, and resistant varieties [2]. The use of disease-resistant cultivars could reduce bacterial wilt in

some tomato-growing regions; however, the durability of a resistant cultivar is affected by many factors, such as regional climate, soil structure, and microbial community of the rhizosphere [12]. Due to concerns over the general use of chemicals in environments [33,43] and the limitations mentioned above, there is an urgent need to develop effective means to manage bacterial wilt disease. Biological control using beneficial microorganisms could be a promising method. The rhizosphere has abundant microorganisms, including *Bacillus* spp. and other antagonistic or plant growth-stimulating bacteria [44], providing great reservoirs for identifying beneficial microorganisms. Therefore, using antagonistic microorganisms in soils could be feasible to control plant soil-borne pathogens.

Many commercially available *Bacillus* spp.-based bio-products are developed to control fungal pathogens. For example, the *B. subtilis* GB03 (also known as *B. velezensis* GB03) strain under the brand name Kodiak has been registered as a fungicide. The *B. pumilus* GB34 strain under the brand name YieldShield (Gustafson, Plano, TX, USA) is known to inhibit the mycelial growth of various fungi. The *B. licheniformis* SB3086 strain under the brand name EcoGuard (Novozymes, Salem, VA, USA) is effective in suppressing many fungal species. The *B. amyloliquefaciens* D747 strain (also known as *B. velezensis* D747) under the brand name Double Nickel 55™ (Certis Biologicals, Columbia, MD, USA) is a broad-spectrum biocontrol agent commonly used to control fungal pathogens, bacterial leaf spot, speck, and fire blight disease [45]. Managing bacterial wilt using the *B. amyloliquefaciens* strain Cas02 [46] or *Pantoea* spp. [47] has recently been explored in greenhouse tests. In this study, we aimed to isolate and identify a bacterium with the antagonistic ability to suppress *R. solanacearum* and demonstrated its effect on controlling bacterial wilt in greenhouse and field trials.

In our research, of 15 heat-resistant bacteria recovered from soils, the PMB01 strain identified as *B. amyloliquefaciens* displayed strong inhibitory activity against *R. solanacearum* on agar plate assays. PMB01 was shown to be highly tolerant to extreme pH, heat, high salt salinity, and various fungicides. These physiological and physical characteristics of PMB01 may increase its durability in the field. Our results also indicate that PMB01 could be used in combination with pesticides for pest management, significantly broadening the utility of PMB01. However, PMB01 is sensitive to copper agents and some antibiotics commonly used to control bacterial diseases, so co-application of PMB01 with these chemicals should be avoided. To produce a large quantity and high quality of fermentation agents for field application, several factors, including nutrient formulation, easy delivery, and maximum bioactivity, should be considered [48]. In this study, we have optimized a fermentation formula to culture *B. amyloliquefaciens* PMB01, revealing that fermentation of PMB01 in a medium containing 1% soybean meal resulted in the strongest antagonistic activity to inhibit the growth of *R. solanacearum* on agar plates. Soybean meal is a common agro-industrial waste and low-cost material [49] and is rich in nitrogen and proteins (44–48%) [50]. Soybean meal has also been shown to increase the production of antimicrobial peptides and the antagonistic activity of fermentation broth [51–53]. Many beneficial microorganisms can hydrolyze fermented soybean meal and produce antimicrobial peptides [53,54]. It is predicted that adding soybean meal to the PMB01 fermentation broth may increase the production of antibacterial peptides, which was validated in the bench fermentation and pilot production of the bioactive PMB01. However, the type of antimicrobial peptides in the fermentation broth needs further investigation. Experimental data have shown that PMB01 could achieve 100% sporulation after 72–96 h fermentation in a 500-L tank for pilot production. Greenhouse assays using the PMB01 fermentation broth have revealed that the disease severity was reduced up to 79.9–83.9% in tomato plants after being treated with undiluted broth or a 100-fold dilution. Applying the culture medium only did not affect the reduction of bacterial wilt. Moreover, no phytotoxicity was found in tomato plants after being treated with PMB01 fermentation broth. However, experimental tests have revealed that the viability of PMB01 in the fermentation broth decreased over time.

For biopesticide application in the field, several measurements must be taken to stabilize the organism and maintain its bioactivity during distribution and storage [55]. In

this study, we compared the shelf life of PMB01 in the wettable powder and fermentation broth. The results revealed that the PMB01 population in the wettable powder decreased by less than 2% at room temperature after two years, whereas the PMB01 population in the fermentation broth decreased by 7%. Thus, PMB01 formulated as wettable powders is more stable during storage and convenient during distribution. Field trials with three different tomato varieties within ten years revealed that PMB01 WP provided a superior effect for controlling bacterial wilt. Moreover, the efficacy of biological control did not differ due to the tomato varieties or climate season in these field trials in the past 10 years. Although applying PMB01 at 200X in some field trials had a moderate impact on the suppression of bacterial wilt, the treatment was significantly different from the water control, demonstrating the effectiveness of PMB01 WP for controlling *R. solanacearum*. Moreover, the control periods of bacterial wilt were sustainable, and the fruit harvesting period could be extended by 35–50 days in ten field trials (data not shown).

Several studies have revealed that antagonistic microorganisms isolated from rhizospheres of diseased plants often perform better in reducing disease incidence. This could be due to their superior ability to colonize plant roots. In addition to antibiosis, root colonizing capacity is one of the critical determinants for the biocontrol efficacy of an antagonist [33,56,57]. Because PMB01 suppressed the severities of bacterial wilt disease in both greenhouse and field trails, it is tempting to speculate that PMB01 could effectively colonize tomato roots, providing strong protection against *R. solanacearum*. The effectiveness of an antagonist against a pathogen is not only affected by the species of antagonists and the methods of mass production but is also influenced by complex environmental factors, all of which could impact the stability of the antagonist and reduce the overall biocontrol effect [58,59]. To achieve effective suppression of bacterial wilt in the field, it is necessary to maintain a stable population of PMB01.

Several mechanisms are known to be responsible for suppressing the growth of a microorganism by a biological control agent. These include the production of antagonistic metabolites, hydrogen cyanide, or siderophores, competition for nutrients and space, parasitism, and induced systemic resistance [20,21,24]. *Bacillus* spp. produce cyclic lipopeptides, such as surfactin, iturin, fengycin/plipastatin, kurstakins, and locillomycin [60]. Thus, *Bacillus* spp. are commonly used as biocontrol agents against diverse phytopathogens. Some bio-agents are known to induce systemic resistance in plants or have plant-growth-promoting activity [61]. Whether PMB01 produces toxic lipopeptides as the mechanism to suppress bacterial wilt disease requires further investigation.

## 5. Conclusions

In this study, we selected and identified the PMB01 strain of *B. amyloliquefaciens* and demonstrated its effectiveness in controlling bacterial wilt disease in ten field trials. We have found that adding 1% soybean meal during fermentation could enhance PMB01 multiplication and greatly reduce the time required for fermentation. Significantly, PMB01 could be stored as wettable powders and remain at nearly 98% viability after 24 months. Field trials on tomato plants have also shown that applying at least a 200-fold dilution of PMB01 could achieve more than a 67% reduction in bacterial wilt disease. Those results indicate a bright future for the development of PMB01 as an effective bio-agent for controlling plant bacterial wilt diseases.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture12101714/s1>, Table S1. Results of 10 field trials in 2014 to 2020.

**Author Contributions:** H.-P.C.: Conceptualization, Methodology, Formal analysis, Writing—Original draft preparation; Y.-C.H.: Formal analysis, Writing—Original draft preparation, Data curation; Y.-H.L.: Original draft preparation, Reviewing and Editing; W.-L.D.: Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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