Developing Fermentation Liquid of *Bacillus amyloliquefaciens* PMB04 to Control Bacterial Leaf Spot of Sweet Pepper

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Abstract: Sweet pepper is an important vegetable in the world. Bacterial leaf spot caused by the pathogen *Xanthomonas perforans,* is a limiting factor that significantly reduces the quality and yield of sweet pepper To control this disease, the use of chemical fungicides is currently the main disease control method. Thus, we want to develop an alternative method by using antagonistic microorganisms. Under this demand, *Bacillus amyloliquefaciens* PMB04 has strong antagonistic effects against pathogens and can inhibit the occurrence of diseases. *B. amyloliquefaciens* PMB04 has the potential for the development of a disease control product. Primarily, PMB04 revealed to contain a strong inhibitory effect against all isolated *X. perforans* strains. In the inoculation assay, the severity of bacterial spot disease on sweet peppers was reduced by PMB04 bacterial suspensions. To increase the convenience of field application for future prospects, the development of PMB04 fermentation liquid was carried out with different ratios of brown sugar and yeast extract in a 30-liter fermentation tank subsequently. Results exhibited that the fermentation liquid of 3-1 formula obtained the highest bacterial population in a 30-liter fermentation tank. The fermentation liquid of 0.5-0.5 formula was the most stable formula under two different conditions in terms of consistent bacterial population and sporulation. In addition, the 200-fold dilution of 3-1 and 0.5-0.5 fermentation liquids revealed best control efficacy on bacterial leaf spot of sweet pepper. Additionally, the results of the 0.5-0.5 fermentation liquid (PMB4FL) with different dilution concentrations also showed that the 200- and 500-fold dilutions had the best control efficacy. To understand the effect of commonly used copper-containing fungicides on sweet peppers on the application of microbial agent PMB4FL, the effects of copper hydroxide and tribasic copper sulfate on the growth of *X. perforans* strains and *B. amyloliquefaciens* PMB04 were assayed. The results exhibited that the above two fungicides did not have any inhibitory effect on the growth of PMB04, but had a strong inhibitory effect on the *X. perforans* strain. In the follow-up control experiment, the treatment of copper hydroxide had no synergistic effect with PMB4FL to control bacterial leaf spot. We concluded that the use of PMB4FL fermentation liquid alone on the leaves can effectively control the occurrence of bacterial leaf spot in sweet pepper crops.

Keywords: agricultural management; antagonistic activity; fermentation formulation; fungicides

Introduction

Sweet pepper is a vegetable crop widely grown in tropical and subtropical areas all over the world, and bacterial leaf spot caused by diverse *Xanthomonas* spp. threatens its production [1]. When bacterial leaf spot of sweet pepper occurs, punctate water-soaked or gangrenous lesions will appear on the leaves and fruits, which will lead to defoliation of the plant and reduction of the economic benefits of fruits in severe cases [2]. To control this disease, agricultural scientists have developed many different methods. In the treatment of seeds, disinfection with hot water or sodium hypochlorite can be used to reduce

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the initial inoculum of pathogenic bacteria [1,3]. On the growing plants, the copper-containing fungicides such as copper hydroxide, tribasic copper sulfate or copper oxide are mainly used, which can effectively reduce the population of pathogenic bacteria in the field. However, under long-term use, there are development of copper-resistant strains that can be isolated from the fields [4,5]. Therefore, other disease control methods that can be combined with existing traditional methods are directions that can be considered. Under this demand, it is extremely feasible to use antagonistic microorganisms to develop microbial agents. To develop the microbial agents for plant disease control, *Bacillus* spp. has been extensively studied due to its ability of producing antagonistic compounds and promoting plant growth. Besides that, this kind of bacteria not only has excellent viability in the field, but also has a good shelf life due to its characteristic of endospore production [6-11]. Among them, *Bacillus amyloliquefaciens* PMB04 has been proven to control fruit blotch of watermelon, black rot of cabbage, and anthracnose of strawberry through its strong antagonistic activity against pathogens [12-14]. Liquid-state fermentation is widely used as it can provide more nutrients and oxygen in a short time [15]. Reports reveal that the production of antagonistic compounds from *Bacillus* spp. can be improved by adjusting the formulation of fermentation liquids, and these fermentation liquids exhibit better biocontrol effect on plant diseases [16,17]. Even in the study of *B. amyloliquefaciens* PMB05, it has been shown that the adjustment of the fermentation liquid formula can also enhance the function of the strain in intensifying plant immunity and exert better disease control ability in the field [18]. Thus, whether this bacterial strain has a good antagonistic effect on the pathogen of bacterial leaf spot on sweet pepper, and whether it can be used to establish a fermentation liquid to prevent the occurrence of the disease is worth to be investigated. In this study, we first confirmed that *B. amyloliquefaciens* PMB04 has good antagonistic activity against different strains of *X. perforans*. Then, the dynamic population changes of *B. amyloliquefaciens* PMB04 on the leaf surface of sweet peppers were also analyzed. Subsequently, the effect of using distinct brown sugar and yeast powder ratio to be regarded as carbon and nitrogen materials, respectively, in the formula of bacterial population and sporulation was analyzed after fermentation. These fermentation liquids were used in the soaking treatment to analyze which formula had the best control effect on bacterial leaf spot of sweet pepper. Moreover, the fermentation liquid with the best control effect was also used to analyze the optimal dilutions for actual application. In order to effectively combine the use of copper-containing fungicides, in addition to analyzing the antibacterial activities of these fungicides against *B. amyloliquefaciens* PMB04 and *X. perforans*, the mixed treatment of fermentation liquid and fungicides was analyzed to determine if there was any synergistic effect on disease control. In this study, we provide evidence that spraying *B. amyloliquefaciens* PMB04 fermentation liquid on leaves of sweet pepper is effective in reducing bacterial leaf spot disease, and it was not affect by applying copper-containing fungicides.

Materials and Methods

Growth conditions for plant and bacteria

The cultivar of sweet pepper (*Capsicum annuum* L.) used in this study was blue star (Known-You Seed Co., Taiwan). The seeds were sown in a 4.5 cm round-hole tray containing sterilized peat moss, and individual 2-week-old seedlings (with 2 true leaves) were transplanted to a 6 cm pot. The seedlings were grown in a growth chamber (Model: F-1200, Hipoint, Kaohsiung, Taiwan) at 28°C under 16 h of light and 8 h of darkness. The 4-week-old seedlings with 4-6 true leaves were used in subsequent experimental analysis.

All the bacterial strains including *B. amyloliquefaciens* PMB04 and *X. perforans* (collected from diseased leaves of sweet pepper) were cultured on nutrient broth agar (NA) plates at 28°C for 48 h. To prepare the bacterial suspension, the colonies on the plate were washed with sterilized 0.1% carboxy methylcellulose (CMC, Sigma, St. Louis, MO, USA) solution and further adjusted its OD_{600} value to 0.3 (about 3.0×10^8 CFU/mL).

Inhibitory assay of B. amyloliquefaciens PMB04 against X. perforans strains

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To investigate whether *B. amyloliquefaciens* PMB04 has broad-spectrum antagonistic effect against different strains of *X. perforans*, the bacterial strains isolated from the field in different regions were used in the assay. The assay was carried out with double layer method. Firstly, a 100 µl of bacterial suspension prepared from each pathogenic bacterial strain was applied in a 5 mL of soft nutrient agar (nutrient broth containing 0.7 % of agar), and then poured the mixture onto NA plate. Two pieces of 8 mm filter paper discs (Advantec, Irvine,California, U.S.A.) were put on the agar plate and dropped 20 µL of PMB04 bacterial suspension or distilled water as negative control onto the filter paper discs. The inhibitory zones were measured at 48 h after incubation at 28 °C. This experiment was carried out with three plates as repeats, and three experimental repetitions were performed for each assay.

Survival of B. amyloliquefaciens PMB04 on the leaf of sweet pepper

To realize whether PMB04 can survive on the leaf surface of sweet pepper, the population changes within one month after leaf spraying were analyzed. The assay was performed with bacterial suspension as describe above. After the bacterial suspension was sprayed on the entire leaves, the plants were placed in the greenhouse to continue growing and sample regularly. For sampling, 1 mL of sterile water was added to every 100 mg of leaf sample for extraction, and then 100 µL of the extract was serially diluted and spread on NA plates to determine the bacterial populations on the leaves. This experiment was carried out twice, and three individual leaves were assayed as repeats.

Effect of B. amyloliquefaciens PMB04 on the control of bacterial leaf spot

To understand the efficacy of *B. amyloliquefaciens* PMB04 on the control of bacterial spot, bacterial suspension or diluted fermentation liquids of *B. amyloliquefaciens* PMB04 was used in this study. The application was carried out by soaking the whole aboveground part of the 4-6 leaves seedlings in the solutions for 30 secs, and the treated seedlings were put in a ventilated place to dry naturally. Then, the inoculation of *X. perforans* XL1 was performed with the bacterial suspension for 30 seconds by the same method. The inoculated plants were wrapped in a transparent plastic bag to keep moisture and placed in a growth chamber at 28 °C. The occurrence of symptoms was observed at 14 days post-inoculation. To calculate the disease severity, the second spread leaf from three individual plants were used to evaluate the disease index. The determination of the disease index is based on the scales of developed symptoms in a 4 cm^2 (2 cm × 2 cm) area (0: no disease symptoms, 1: less than 5 yellowing spots, 2: more than five yellowing spots, 3: more than 5 lesions with necrosis symptom, 4: healed necrosis lesions, 5: healed necrosis lesions with shot hole.) The disease severity of each leaf was calculated using the following formula: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5)/ (5 \times \text{number of leaves})] \times 100\%$ [19]. The disease severities were calculated from five sets of plants in each treatment.

Effects of different fermentation formula on bacterial population and sporulation of 30 L harvested fermentation liquid

To understand the effect of fermentation formula on bacterial population and sporulation of *B. amyloliquefaciens* PMB04 in fermentation liquids of, the assay was carried out in a 30-liter tank (BTF-B30L, Biotop Process & Equipment Inc., Nantou County, Taiwan). To begin the fermentation process, 2% volume of overnight culture of *B. amyloliquefaciens* PMB04 prepared from Luria–Bertani (LB) broth was added into the sterilized fermentation formula and further incubated at 37°C under 120 rpm for 5 days [20]. The fermentation formula (3-1, 2-1, and 1-1) was tested by adjusting the weight percentage of granulated sugar from 3% to 1% in the case of 1% yeast extract (Sunright, New Taipei City, Taiwan). In addition, a formula (0.5-0.5) composed of 0.5% brown sugar and 0.5% yeast extract was also used for analysis. In the analysis of the bacterial population of distinct fermentation liquids, samples were taken and determined by serial dilution method. In terms of the sporulation, the analysis was performed according the standard method [21]. Briefly, a 10 mL of each fermentation liquid was taken and incubated at 70°C for 30 mins

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in the water bath. The number of survived endospores in the fermentation liquid was also determined by serial dilution. The sporulation of fermentation liquid was calculated using the following formula: Sporulation (%) = (the number of endospore/ total bacterial population) × 100%. Each fermentation liquid was sampled three times as repeats, and a total of 3 independent analysis were performed.

Control effect of B. amyloliquefaciens PMB04 fermentation liquids on bacterial spot of sweet pepper

To understand the effects of formulations difference on the control of bacterial leaf spot disease on sweet pepper, distinct *B. amyloliquefaciens* PMB04 fermentation liquid was applied in the inoculation assay. For each treatment, the 4-week old seedlings were soaked into 200-fold dilution of fermentation liquid for 30 seconds. The 0.1% of CMC was used as the blank treatment. After the water film on the leaves was dried, the treated seedlings were then soaked in the bacterial suspension of *X. perforans* XL1 for 30 seconds. Then, the disease severity was determined as described above at 14 day-post- inoculation.

Sensitivity of B. amyloliquefaciens PMB04 and X. perforans XL1 to copper-containing fungicides

To evaluate whether commonly used copper-containing fungicides have the potential to be used together with fermentation liquid in the field, the effects of copper-containing fungicides on the growth of *B. amyloliquefaciens* PMB04 and *X. perforans* XL1 were assayed in nutrient broth. Before assay, bacterial suspensions of *B. amyloliquefaciens* PMB04 and *X. perforans* XL1 at OD_{600} 0.3 were prepared. A 100 µL of bacterial suspension was added into a 5 ml of nutrient broth containing 0.54 mg ai mL^{-1} of tribasic copper sulfate (NUFARM GmbH & Co KG, St. Peter-Strass, Australia, 500×) or 0.27 mg ai mL^{-1} of copper hydroxide (Corteva agriscience, Houston, U.S.A., 2000×). The OD_{600} values was determined after the mixture was incubated at 28 °C under 200 rpm for 12 h and 24 h. The experiment was performed with 3 repeats for each treatment.

Effect of B. amyloliquefaciens PMB04 fermentation liquid filtrate against X. perforans

To realize whether the fermentation liquid of *B. amyloliquefaciens* PMB04 has a better inhibitory effect to inhibit *X. perofrans* than the culture broth, this experiment was conducted with the filtrates obtained from the fermentation liquid (0.5-0.5, named PMB4FL) and culture broth for analysis. To obtain the filtrates, all the materials were centrifuged at 8000 ×g for 10 min and further filtered with a 0.22 µm filter. And a 500 µL of filtrate was added into 500 µL of *X. perforans* XL1 bacterial suspension. After incubation at 28 °C for 8 h, 1 mL of the mixture was transferred to a new microtube and stained with 1.5 µL of SYTO 9 (Thermo, Waltham, Massachusetts,U.S.A) in the dark for 30 min. The images were observed under specific filter (Excitation/ Emission: 465-495 nm/515-555 nm) under fluorescent microscope (Leica, Wetzlar, Germany). The images were used to calculate the fluorescence intensities by using the ImageJ software (https://imagej.nih.gov/ij/). In each treatment, 10 images were taken as repeats.

Effect of copper hydroxide on B. amyloliquefaciens PMB04 fermentation liquid in the control of bacterial leaf spot of sweet pepper

To understand whether copper-containing fungicides would affect the control effect of *B. amyloliquefaciens* PMB04 fermentation liquid to bacterial leaf spot disease on sweet pepper, the copper hydroxide copper agent and the fermentation liquid (PMB4FL) were used in the assay. Before inoculation, the 4-week-old seedlings were soaked in the 200-fold diluted PMB4FL, 2000-fold diluted copper hydroxide, or the mixture containing 200-fold diluted PMB4FL and 2000-fold diluted copper hydroxide for 30 seconds. The treatment with 0.1% of CMC was used as the blank treatment. After the water film on the leaves were dried, the treated seedlings were then soaked in the bacterial suspension of *X. perforans* XL1 for 30 seconds to perform the inoculation. The disease severity was determined as described above at 14 day-post-inoculation.

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Data analysis

Statistical analysis was performed using SPSS Statistics software for Windows, version 25 (IBM Corp, Armonk, NY, USA). Analysis of variance (ANOVA) and post hoc tests (Tukey's HSD) were used to analyze the significant differences between treatments in the assays ($p < 0.05$).

Results

Inhibitory effect of Bacillus amyloliquefaciens PMB04 against Xanthomonas perforans strains

To test whether *B. amyloliquefaciens* PMB04 can broadly inhibit the strains of *X. perforans* (isolated from diseased tissue of sweet pepper obtained from different fields) a confrontation assay was performed. Results showed that all *X. perforans* strains were inhibited by *B. amyloliquefaciens* PMB04, especially the XL1, XL2, XL4 and G1 strains (Figure 1).

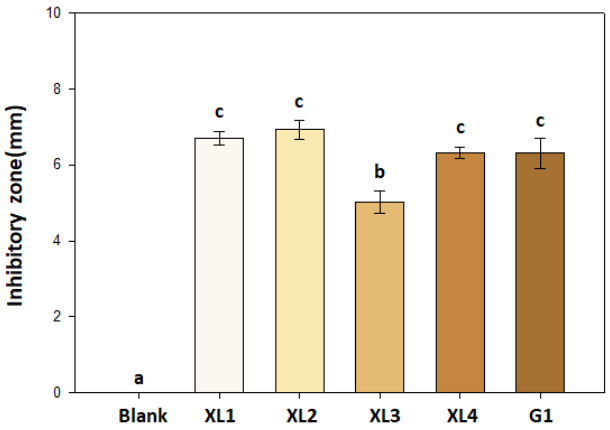

Figure 1. Inhibitory effect of *Bacillus amyloliquefaciens* PMB04 against *Xanthomonas perforans* strains. The confrontation assay was performed with the double-layer agar method. The top layer was mixed with the bacterial suspension of each *X. perforans* strains and then poured on the nutrient agar plates. After a paper disc placed on the top layer, a 20 μL of *B. amyloliquefaciens* PMB04 bacterial suspension was applied on the paper disc. Blank indicates the treatment with sterilized water as negative control. Different letters above columns indicated significant differences between different bacterial pathogens based on Tukey's HSD test ($p < 0.05$).

Survival of B. amyloliquefaciens PMB04 on the leaves of sweet pepper

To evaluate the survival ability of *B. amyloliquefaciens* PMB04 on the leaves of sweet pepper, its dynamic changes on bacterial population was determined after spraying with the bacterial suspension. Result showed that the initial population of PMB04 was 4.22×10^6 CFU/ g leaf, and the bacterial population after three weeks was 4.38×106 CFU/ g leaf, and there was no significant difference between the populations at these two time points (Figure 2).

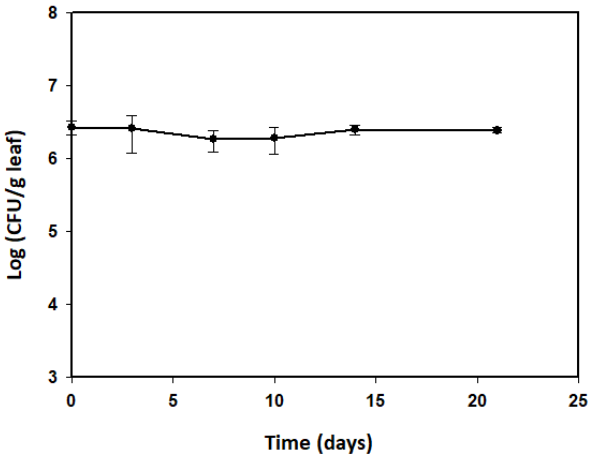

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Figure 2. Dynamic changes on bacterial population of *Bacillus amyloliquefaciens* PMB04 on the leaves of sweet pepper in greenhouse. Before treatment, the bacterial suspension of *B. amyloliquefaciens* PMB04 was adjusted its OD_{600} to 0.3 in 0.1% of carboxy methylcellulose. After spraying the bacterial suspension on the leaves of sweet pepper, the seedlings were placed in greenhouse for 21 days. The bacterial population was determined by serial dilution to count the CFU per gram of leaf tissue.

Control efficacy of Bacillus amyloliquefaciens PMB04 bacterial suspension to bacterial leaf spot disease in sweet pepper

To confirm whether the strong antagonistic activity of *B. amyloliquefaciens* PMB04 to *X. perforans* strains would be able to control bacterial leaf spot, its bacterial suspension was pretreated on sweet pepper. The results showed that the symptom development of bacterial leaf spot was inhibited by *B. amyloliquefaciens* PMB04. In addition, the disease severity of PMB04 treatment was significantly reduced to 48.33%, compared to 73.33% of blank treatment. Meanwhile, the control efficacy was 34.10% (Figure 3).

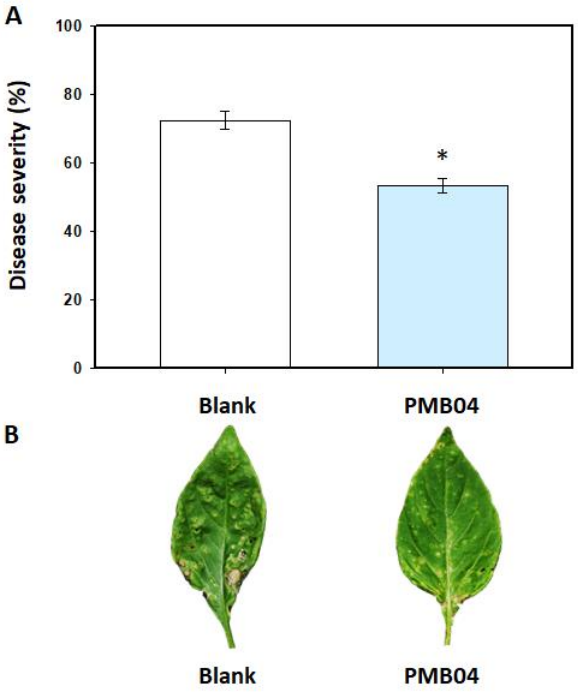

Figure 3. Effect of *Bacillus amyloliquefaciens* PMB04 bacterial suspension on the control of bacterial leaf spot in sweet pepper. Before inoculation, the seedlings were soaked in a bacterial suspension of *B. amyloliquefaciens* PMB04. Then, the air-dried seedlings were soaked in a bacterial suspension of X. perforans XL1 for the inoculation. Panel A reveals the disease severity after inoculation. The * indicate a significant difference compared with the blank treatment, as assessed using a t-test ($p < 0.05$). Panel B shows the visual symptoms of bacterial leaf spot reduced by *B. amyloliquefaciens* PMB04 on sweet pepper.

Effects of formulated differences on the population and sporulation of Bacillus amyloliquefaciens PMB04 fermentation liquids from a 30-liter fermenter

To realize the effect of brown sugar concentrations in the formulations on the fermentation of *B. amyloliquefaciens* PMB04, 3% to 1% brown sugar was applied in the basal formula with 1% of yeast extract to evaluate the cell production and sporulation of *B. amyloliquefaciens* PMB04 in fermentation liquids. Results exhibited that the cell population of *B. amyloliquefaciens* PMB04 in 3-1, 2-1, and 1-1 fermentation liquids were 1.00×10^9, 9.01×10^8, and 3.50×10^8 CFU/mL, respectively. In addition, in 0.5-0.5 fermentation liquid, cell population was 4.83×10^8 CFU/mL. The 3-1 formulation exhibited highest population of PMB04 than other formulations in the fermentation liquid. Moreover, the survival cells after heating exhibited that the sporulation ratios of *B. amyloliquefaciens* PMB04 reached 100% in all the fermentation liquids (Table 1).

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Table 1. Effect of fermentation formulations composed with brown sugar and yeast extract on population and sporulation of *Bacillus amyloliquefaciens* PMB04 in a 30-Liter fermentation tank.

Formulation	Cells (Log CFU/ml)	Endospores (Log CFU/ml)	Sporulation (%)
3-1[1]	9.00±0.41[a]	9.29±0.41[a]	100.00
2-1	8.91±0.21[ab]	9.09±0.33[ab]	100.00
1-1	8.54±0.06[b]	8.47±0.08[b]	99.14
0.5-0.5	8.68±0.01[b]	8.75±0.08[b]	100.00

[1]The numerical code indicates the proportion of brown sugar and yeast extract in the fermentation formulation. Different letters in the same column indicates significant differences between different formulations based on Tukey's HSD test ($p < 0.05$).

Effects of B. amyloliquefaciens PMB04 fermentation liquids on the control of bacterial spot

To evaluate distinct *B. amyloliquefaciens* PMB04 fermentation liquids on the control of bacterial leaf spot, 200-fold dilution of each fermentation liquid was first used in the inoculation assay. Results showed that all of the *B. amyloliquefaciens* PMB04 fermentation liquids reduced the occurrence of bacterial leaf spot significantly. While compared to the disease severity in the blank treatment (76.67%), that with 3-1, 2-1, and 1-1 fermentation liquids were 44.44%, 47.50%, 55.56% and 42.50%, respectively (Figure 4). The control efficacy of 3-1, 2-1, and 1-1 fermentation liquids were 42.03%, 38.04%, 27.53% and 44.57%. The 3-1 and 0.5-0.5 fermentation liquids exhibited the best control efficacy on bacterial spot of sweet pepper.

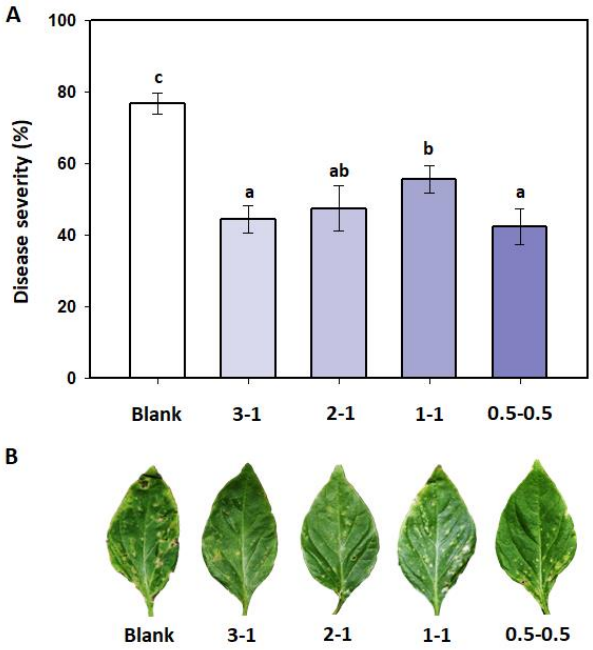

Figure 4. Effect of *Bacillus amyloliquefaciens* PMB04 fermentation liquids from distinct formulations on the control of bacterial leaf spot in sweet pepper. The assay was conducted by soaking method with seedlings in each diluted fermentation liquid. Blank indicates the blank treatment with water as negative control; 3-1, 2-1, 1-1, and 0.5-0.5 indicate the treatment with 200× dilution of fermentation liquids from formulations consisting of different proportions of brown sugar and yeast extract. Panel A reveals the disease severity after inoculation. The different letters above columns indicate significant differences between different treatments based on Tukey's HSD test ($p < 0.05$). Panel B shows the visual symptoms of bacterial leaf spot reduced by *B. amyloliquefaciens* PMB04 fermentation liquids on sweet pepper.

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Since the 0.5-0.5 fermentation liquid (PMB4FL) has shown superior control efficacy on bacterial spot of sweet pepper, the assay was further carried out by dilutions with different concentrations. The results showed that treatments with 200 ×, 500 × and 1000 × dilutions of PMB4FL reduced the occurrence of bacterial leaf spot, with their disease severities being 46.67%, 54.67% and 63.75%, respectively (Figure 5). Furthermore, the control efficacy of 200×, 500× and 1000× dilutions had disease severity of 39.13%, 28.70% and 16.85%, respectively. Compared to the blank treatment, the 200× dilution was the most effective treatment in disease control.

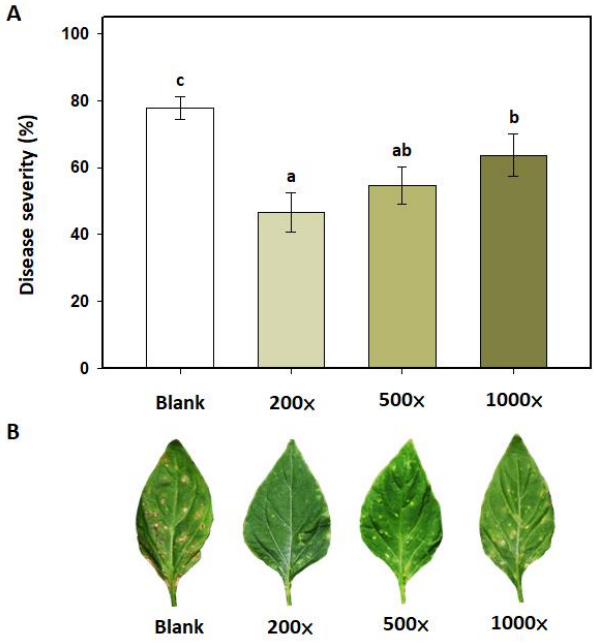

Figure 5. Effect of distinct dilutions of PMB4FL fermentation liquid on the control of bacterial leaf spot in sweet pepper. The assay was conducted by soaking method with seedlings in each dilution of PMB4FL. Blank indicates the blank treatment with water as negative control; the treatments were carried out with 200×, 500×, and 1000× dilutions of PMB4FL. Panel A reveals the disease severity after inoculation. The different letters above columns indicate significant differences between different treatments based on Tukey's HSD test ($p < 0.05$). Panel B shows the visual symptoms of bacterial leaf spot reduced by dilution of PMB4FL on sweet pepper.

Effect of B. amyloliquefaciens PMB04 fermentation filtrate on the survival of X. perforans cells

To confirm whether the *B. amyloliquefaciens* PMB04 fermentation liquid with the best control efficacy would affect the cell survival of *X. perforans*, the filtrates from PMB4FL or LB was applied for analysis. The green fluorescence indicating surviving cells was not notably different in the treatment of the LB culture filtrate compared with the blank at 8 h after treatment, but there was less fluorescence in the treatment of the PMB4FL filtrate (Figure 6A). After quantification, results showed that the relative fluorescence of the PMB4FL filtrate treatment was significantly lower than that of the blank and LB culture filtrate treatment (Figure 6B).

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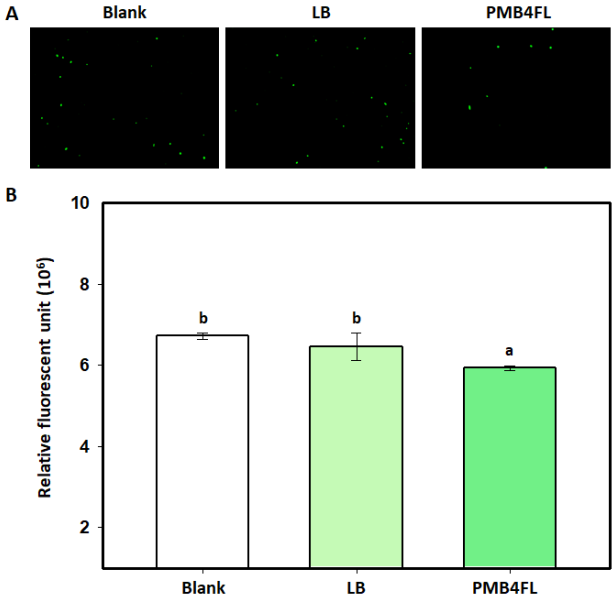

Figure 6. Effects of *Bacillus amyloliquefaciens* PMB04 filtrates from PMB4FL and culture broth on the cell viability of *Xanthomonas perforans* XL1. The assay was performed by applying a filtrate from PMB4FL and LB culture of *Bacillus amyloliquefaciens* PMB04 in the nutrient broth with *X. perforans* XL1. After incubation at 28 °C under 200 rpm for 8 hours, the SYTO 9 was used to stained living cells. Panel A shows the fluorescent image of living cells of *X. perforans* XL1 under different treatments at 8 h after treatment. Panel B indicated the relative fluorescent unit (RFU) determined at 485/525 nm for SYTO 9 at 8 h after treatment. Different letters above columns indicate significant differences between treatments based on Tukey's HSD test ($p < 0.05$).

Inhibitory effect of copper-containing fungicides against B. amyloliquefaciens PMB04 and X. perforans XL1

To understand the potential impact of copper-containing fungicides commonly used to control bacterial spot of Solanaceae crops on the application of microbial agents in the field, their effects on bacterial growth of *B. amyloliquefaciens* PMB04 and *X. perforans* were first determined for evaluation. Results showed that the treatment of tribasic copper sulfate or copper hydroxide had no effect on the growth of *B. amyloliquefaciens* PMB04 compared to the blank treatment. However, both tribasic copper sulfate and copper hydroxide have a superior inhibitory effect on the growth of *X. perforans* XL1 (Table 2).

Table 2. Effect of copper-containing fungicides on the growth of *Bacillus amyloliquefaciens* PMB04 and *Xanthomonas perforans* XL1 against in nutrient broth.

Strain	Fungicide	OD_{600}	
		12 h	24 h
B. amyloliquefaciens PMB04			
	Blank	0.180^{a}	0.367^{a}
	Tribasic copper sulfate	0.161^{a}	0.395^{ab}
	Copper hydroxide	0.175^{a}	0.420^{b}
X. perforans XL1			
	Blank	0.123^{a}	0.257^{a}
	Tribasic copper sulfate	0.043^{b}	0.162^{c}
	Copper hydroxide	0.041^{b}	0.193^{b}

Different letters in the same column indicates significant differences between treatments based on Tukey's HSD test ($p < 0.05$).

Effects of copper hydroxide on PMB4FL in the control of bacterial leaf spot in sweet pepper

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To understand the effect of copper-containing fungicides on the control of PMB4FL fermentation liquid, copper hydroxide was used as a model for control analysis. Results showed that the disease severity of the blank treatment was 77.78%, the disease severities of 200× dilution of PMB4FL alone, 2000× dilution of copper hydroxide alone, and the mixture containing 200× dilution of PMB4FL and 2000× dilution of copper hydroxide were 50.00%, 68.75% and 55.56%, respectively (Figure 7). Only the treatment with PMB4FL alone and the treatment with the mixture of PMB4FL and copper hydroxide could significantly reduce the occurrence of disease, which brought 34.78% and 27.54% of control efficacy. Although the copper hydroxide treatment has a 10.33% of control efficacy, there is no significant difference in the disease severity compared with the blank treatment.

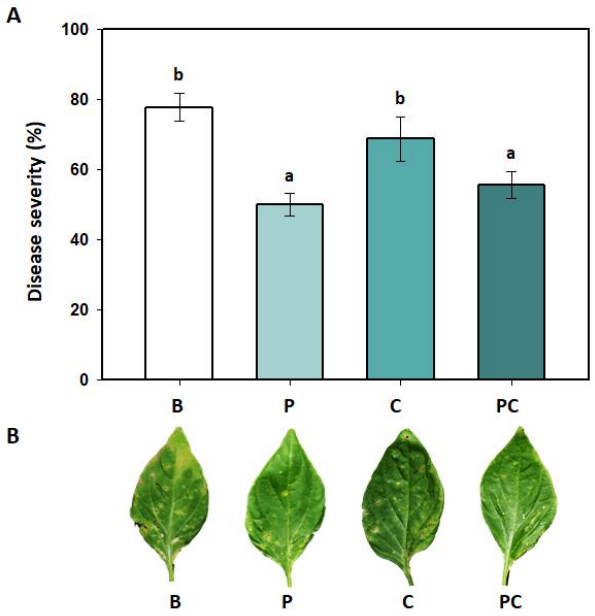

Figure 7. Effect of copper hydroxide on control efficacy of PMB4FL to bacterial leaf spot on sweet pepper. The assay was conducted by soaking method with seedlings in each solution. B indicates the blank treatment with water as negative control; P indicates the treatment with 200× dilution of PMB4FL; C indicates the treatment with 2000× dilution of copper hydroxide; and PC indicates the treatment with the mixture containing 200× dilution of PMB4FL and 2000× dilution of copper hydroxide. Panel A shows the disease severity at 14-day post-inoculation. Different letters above columns indicate significant differences between treatments based on Tukey's HSD test ($p < 0.05$). Panel B shows the development of bacterial leaf spot disease in sweet pepper among different treatments.

Discussion

With the increasing attention to food safety, the use of beneficial microorganisms to control plant disease is accepted by the public as a way to produce safe agricultural products. It is also a very important topic in agricultural science today. Among the beneficial microorganisms that can be applied, *Bacillus* spp. has attracted much attention because of its ability on producing endospores to withstand adverse environmental conditions such as high and low temperatures, chemicals, and ultraviolet light [22,23]. In addition, many bacterial strains in this genus have been reported to produce a variety of secondary metabolites to destroy the cells of pathogenic bacteria or enhance plant defense responses to help plants resist diseases [24-28]. Currently, many studies in the world have proved that *Bacillus* spp. strains can effectively reduce the occurrence of various bacterial and fungal diseases by its antagonistic activity [6,29-33], indicating that the metabolites produced by *Bacillus* spp. are an important mechanism for disease control. In previous reports, it was proved that the antagonism of *Bacillus amyloliquefaciens* PMB04 to the plant pathogens *Acidovorax citrulli*, *Xanthomonas campestris* pv. *campestris* and *Colletotrichum gloeosporioides*, can be effective to control watermelon fruit blotch, cabbage black rot, strawberry anthracnose and mango anthracnose respectively. Since sweet pepper is an important vegetable

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crop in central and southern Taiwan, the occurrence of bacterial leaf spot disease caused by *Xanthomonas perforans* often causes tremendous losses to farmers. Therefore, this study intends to explore the control effect of *B. amyloliquefaciens* PMB04 on bacterial leaf spot of sweet pepper crops and to investigate whether the subsequent adjustment of the fermentation formula can further increase the control effect of this disease. In this study, PMB04 showed superior antagonistic activity against most of the *X. perforans* strains isolated from the field. Similarly, it was also proved that *B. amyloliquefaciens* PMB04 can survive on the leaves of sweet pepper for more than 21 days and can maintain a 100% survival rate under greenhouse conditions. In many reports, *B. amyloliquefaciens* strains has been demonstrated that this species has good ability on plant colonization [34-36]. In this study, it was shown that *B. amyloliquefaciens* PMB04 has good survival ability on sweet pepper leaf surface. From the aforementioned results, it can be speculated that *B. amyloliquefaciens* PMB04 should have the potential to control bacterial leaf spot of sweet pepper crops. In the further biocontrol assay by using the bacterial suspension of *B. amyloliquefaciens* PMB04, we demonstrated that *B. amyloliquefaciens* PMB04 can significantly reduce the occurrence of bacterial leaf spot.

The application potential of beneficial microorganisms is often limited due to insufficient number of bacteria or time-consuming cultivation. Using fermentation technology for cultivation can not only increase the number of microbial populations rapidly, but also increase the production of enzymes or antagonistic substances [16,37-40]. Among them, liquid fermentation can provide nutrients and oxygen supply directly to microbial strains with a shorter time frame, so it is widely used [41]. In this study, brown sugar and yeast powder were used as the basis of the formulation to explore the effects of different proportions of the formulation on the control of *B. amyloliquefaciens* PMB04 against bacterial leaf spot of sweet pepper. Our results showed that the 4 formulations designed in this study, the sporulation rate of all fermentation liquid reached 100%, among which the formula 3-1 could obtain the highest bacterial population. Based on these results, we speculated that the bacterial population can indeed be increased under the higher sugar supply, and most of the supply of these nutrient sources can be used up by *B. amyloliquefaciens* PMB04 to enter the process of endospore formation.

Further use of *B. amyloliquefaciens* PMB04 fermentation liquids from different formulations to evaluate the control efficacy on bacterial leaf spot in sweet pepper showed that all formulations could significantly reduce the occurrence of bacterial leaf spot, and there were no differences between formulations in the control efficacy. The result indicated that even if the 3-1 formulation could increase the bacterial population after fermentation, such an increase cannot improve the control efficacy on diseases. From this result, it can be speculated that the control efficacy is mainly due to the antagonistic compounds in the fermentation liquids, and it can be further speculated that the formula (0.5-0.5) of the lowest nutrient sources used in this study can effectively produce enough antagonistic substances to control bacterial leaf spot disease. In this study, the follow-up analysis of the 0.5-0.5 formula (PMB4FL) also proved that the PMB4FL fermentation liquid can effectively control the occurrence of bacterial leaf spot by using 200- or 500-fold dilution. This result is similar to the recommended dilutions for tomato bacterial wilt and lemon canker in our previous fermentations prepared with other *B. amyloliquefaciens* strains [18,42]. In addition, we used the technique of living cell staining to prove that the number of *X. perforans* living cells was reduced by the filtrate of PMB4FL, and this result can be further speculated that the *X. perforans* cells can be killed by the antagonistic substances in the fermentation liquid. Many studies have shown that lipopeptide compounds such as iturin, fengycin and surfactin are widely found in *Bacillus* spp. The inhibitory activities of these compounds against different bacterial pathogens all play an important role in disease control [7,43,44]. After the whole genome sequence of *B. amyloliquefaciens* PMB04 was sequenced, the preliminary prediction results of secondary metabolites by antiSMASH showed that this strain had the existence of related genes for producing bacillibactin, bacillaene, bacilysin and fengycin (data not shown). However, as for what kind of antagonistic substances still need to be further investigations.

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To increase the opportunities of the actual application of microbial agents in the field, it is necessary to consider how to cooperate it with the use of conventional fungicides in the field. Although the causal agents of bacterial leaf spot, *X. perforans*, may have copper resistance genes to exhibit copper resistance [45], copper-based fungicides are still the main recommended treatment of bacterial leaf spot of Solanaceae plants.

The results of the analysis of copper hydroxide and tribasic copper sulfate in NB culture medium showed that these two fungicides still had significant inhibitory effects on the growth of tested *X. perforans* strain, but they had no inhibitory effect on the growth of *B. amyloliquefaciens* PMB04. The results of the control efficacy assay showed that copper hydroxide had no significant control effect on sweet pepper bacterial leaf spot. Previously, Obradovic *et al.* demonstrate that the bacterial leaf spot disease can be reduced by the application with copper hydroxide [46]. We hypothesize that the main reason for the difference between our results and the literature may be due to the differences in bacterial strains. In addition, we speculated that although the growth of *X. perforans* can be inhibited in liquid medium, it may not be able to show the ideal inhibitory activity on the leaves to reduce the disease. However, the addition of copper hydroxide could not increase or decrease the effect of PMB4FL fermentation liquid on the control of bacterial leaf spot. The result is similar to that done by Korsten *et al*., that *Bacillus subtilis* and copper oxychloride were used together to control post-harvest diseases of avocado, and copper oxychloride could not increase the control effect of *Bacillus subtilis* [47]. From these results, it can be inferred that *B. amyloliquefaciens* PMB04 should be tolerant to copper, and the PMB4FL fermentation liquid can be applied alone in the field in the future to exert the effect of controlling bacterial leaf spot of sweet pepper.

Conclusion

In this study, we confirmed that *B. amyloliquefaciens* PMB04 has a good antagonistic activity against *X. perforans* strains. At the same time, we have also established a PMB4FL fermentation liquid with very streamlined nutrient sources and effective control of bacterial leaf spot in sweet pepper. More importantly, this study proves that the application of PMB4FL fermentation liquid will not be impaired by the application of a copper-containing fungicide, and its control effect has the potential to be applied in the field.

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References

1. Potnis, N.; Timilsina, S.; Strayer, A.; Shantharaj, D.; Barak, J.D.; Paret, M.L.; Vallad, G.E.; Jones, J.B. Bacterial spot of tomato and pepper: diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge. *Mol. Plant. Pathol.* **2015**, *16*, 907-920, doi:10.1111/mpp.12244.
2. Roach, R.; Mann, R.; Gambley, C.G.; Shivas, R.G.; Rodoni, B. Identification of *Xanthomonas* species associated with bacterial leaf spot of tomato, capsicum and chilli crops in eastern Australia. *Eur. J. Plant Pathol.* **2017**, *150*, 595-608, doi:10.1007/s10658-017-1303-9.
3. Sanogo, S.; Clary, M. *Bacterial leaf spot of chile pepper: a short guide for growers*; New Mexico State University, College of Agriculture and Home Economics …: 2008.

4. Hsiao, Y.-M.; Liu, Y.-F.; Lee, P.-Y.; Hsu, P.-C.; Tseng, S.-Y.; Pan, Y.-C. Functional characterization of copA gene encoding multi-copper oxidase in *Xanthomonas campestris* pv. *campestris*. *J. Agric. Food Chem.* **2011**, *59*, 9290-9302, doi:doi: 10.1021/jf2024006.
5. Voloudakis, A.E.; Reignier, T.M.; Cooksey, D.A. Regulation of Resistance to Copper in *Xanthomonas axonopodis* pv. *vesicatoria*. *Appl. Environ. Microbiol.* **2005**, *71*, 782-789, doi:10.1128/aem.71.2.782-789.2005.
6. Almoneafy, A.A.; Kakar, K.U.; Nawaz, Z.; Li, B.; saand, M.A.; Chun-lan, Y.; Xie, G.-L. Tomato plant growth promotion and antibacterial related-mechanisms of four rhizobacterial *Bacillus* strains against *Ralstonia solanacearum*. *Symbiosis* **2014**, *63*, 59-70, doi:10.1007/s13199-014-0288-9.
7. Cao, Y.; Pi, H.; Chandrangsu, P.; Li, Y.; Wang, Y.; Zhou, H.; Xiong, H.; Helmann, J.D.; Cai, Y. Antagonism of two plant-growth promoting *Bacillus velezensis* isolates against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci. Rep.* **2018**, *8*, 1-14, doi:10.1038/s41598-018-22782-z.
8. Olishevska, S.; Nickzad, A.; Déziel, E. *Bacillus* and *Paenibacillus* secreted polyketides and peptides involved in controlling human and plant pathogens. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 1189-1215, doi:10.1007/s00253-018-9541-0.
9. Ongena, M.; Jacques, P. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* **2008**, *16*, 115-125.
10. Setlow, P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J. Appl. Microbiol.* **2006**, *101*, 514-525, doi:10.1111/j.1365-2672.2005.02736.x.
11. Vardharajula, S.; Zulfikar Ali, S.; Grover, M.; Reddy, G.; Bandi, V. Drought-tolerant plant growth promoting *Bacillus* spp.: effect on growth, osmolytes, and antioxidant status of maize under drought stress. *J. Plant Interact.* **2011**, *6*, 1-14, doi:10.1080/17429145.2010.535178.
12. Chang, J.-J.; Wu, P.-Y.; Lin, Y.-N.; Deng, W.-L.; Lin, Y.-H. Intensification of PAMP-triggered immunity in watermelon by *Bacillus* spp. strains as a strategy for controlling bacterial fruit blotch disease. *J. Plant Med.* **2019**, *61*, 39-48.
13. Li'aini, A.S.; Lin, Y.-H.; Huang, T.-C.; Sulistyowati, L. Application of *Bacillus amyloliquefaciens* to control black rot disease on cabbage caused by *Xanthomonas campestris* pv. *campestris*. *J. Plant Med.* **2017**, *59*, 39-44, doi:DOI:10.6716/JPM.201709_59(3).0005.
14. Wu, Y.-M.; Chen, X.; Wang, F.; Hsiao, C.-Y.; Yang, C.-Y.; Lin, S.-T.; Wu, L.-H.; Chen, Y.-K.; Liang, Y.-S.; Lin, Y.-H. *Bacillus amyloliquefaciens* strains control strawberry anthracnose through antagonistic activity and plant immune response intensification. *Biol. Control* **2021**, *157*, 104592.
15. Zeriouh, H., Romero, D., García-Gutiérrez, L., Cazorla, F. M., de Vicente, A., and Pérez-García, A. The iturin-like lipopeptides are essential components in the biological control arsenal of *Bacillus subtilis* against bacterial diseases of cucurbits. *MPMI* **2011**, *24*, 1540-1552.
16. Ahsan, T.; Zang, C.; Yu, S.; Pei, X.; Xie, J.; Lin, Y.; Liu, X.; Liang, C. Screening, and Optimization of Fermentation Medium to Produce Secondary Metabolites from Bacillus amyloliquefaciens, for the Biocontrol of Early Leaf Spot Disease, and Growth Promoting Effects on Peanut (Arachis hypogaea L.). *J. Fungi* **2022**, *8*, 1223, doi:10.3390/jof8111223.
17. Zhao, B.; Cao, X.; Cai, Z.; Zhang, L.; Li, D.; Zhang, H.; Li, S.; Sun, X. Improving suppressive activity of compost on phytopathogenic microbes by inoculation of antagonistic microorganisms for secondary fermentation. *Bioresour. Technol.* **2023**, *367*, 128288, doi:doi: 10.1016/j.biortech.2022.128288.
18. Lin, K.-W.; Liang, Y.-S.; Hsiao, C.-Y.; Wang, F.; Huang, T.-P.; Lin, Y.-H. Application of fermentation broth of *Bacillus amyloliquefaciens* PMB05 to control bacterial canker disease on lemon. *J. Plant Med.* **2021**, *63*, 17-26.
19. Chuang, C.-Y.; Lin, S.-T.; Li, A.-T.; Li, S.-H.; Hsiao, C.-Y.; Lin, Y.-H. *Bacillus amyloliquefaciens* PMB05 Increases Resistance to Bacterial Wilt by Activating Mitogen-Activated Protein Kinase and Reactive Oxygen Species Pathway Crosstalk in *Arabidopsis thaliana*. *Phytopathology* **2022**.
20. Liang, Y.-S.; Fu, J.-Y.; Chao, S.-H.; Tzean, Y.; Hsiao, C.-Y.; Yang, Y.-Y.; Chen, Y.-K.; Lin, Y.-H. Postharvest Application of Bacillus amyloliquefaciens PMB04 Fermentation Broth Reduces Anthracnose Occurrence in Mango Fruit. *Agriculture* **2022**, *12*, 1646, doi:10.3390/agriculture12101646.
21. Gould, G.; Wrighton, C. Limitations of the initiation of germination of bacterial spores as a spore control procedure. . *J. Appl. Bacteriol.* **1968**, *31*, 357-366.
22. Lin, K.-W., Liang, Y.-S., Hsiao, C.-Y., Wang, F., Huang, T.-P., and Lin, Y.-H. Application of fermentation broth of Bacillus amyloliquefaciens PMB05 to control bacterial canker disease on lemon. *J. Plant Med.* **2021**, *63*, 17-26.
23. Nicholson, W.L.; Munakata, N.; Horneck, G.; Melosh, H.J.; Setlow, P. Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews* **2000**, *64*, 548-572, doi:doi:10.1128/mmbr.64.3.548-572.2000.
24. Gase, K.; Ferretti, J.J.; Primeaux, C.; McShan, W.M. Identification, Cloning, and Expression of the CAMP factor gene (cfa) of Group A Streptococci. *Infection and Immunity* **1999**, *67*, 4725-4731, doi:doi:10.1128/iai.67.9.4725-4731.1999.
25. Ghelardi, E.; Salvetti, S.; Ceragioli, M.; Gueye, S.A.; Celandroni, F.; Senesi, S. Contribution of Surfactin and SwrA to Flagellin Expression, Swimming, and Surface Motility in Bacillus subtilis. *Applied and Environmental Microbiology* **2012**, *78*, 6540-6544, doi:doi:10.1128/AEM.01341-12.
26. Wu, L.; Wu, H.; Chen, L.; Yu, X.; Borriss, R.; Gao, X. Difficidin and bacilysin from Bacillus amyloliquefaciens FZB42 have antibacterial activity against Xanthomonas oryzae rice pathogens. *Scientific Reports* **2015**, *5*, 12975, doi:10.1038/srep12975.
27. Wu, Z.; Huang, Y.; Li, Y.; Dong, J.; Liu, X.; Li, C. Biocontrol of Rhizoctonia solani via Induction of the Defense Mechanism and Antimicrobial Compounds Produced by Bacillus subtilis SL-44 on Pepper (Capsicum annuum L.). *Frontiers in Microbiology* **2019**, *10*, doi:10.3389/fmicb.2019.02676.

28. Yi, H.-S.; Ahn, Y.-R.; Song, G.C.; Ghim, S.-Y.; Lee, S.; Lee, G.; Ryu, C.-M. Impact of a Bacterial Volatile 2,3-Butanediol on Bacillus subtilis Rhizosphere Robustness. *Frontiers in Microbiology* **2016**, *7*, doi:10.3389/fmicb.2016.00993.
29. Chen, D.; Liu, X.; Li, C.; Tian, W.; Shen, Q.; Shen, B. Isolation of Bacillus amyloliquefaciens S20 and its application in control of eggplant bacterial wilt. *Journal of Environmental Management* **2014**, *137*, 120-127, doi:https://doi.org/10.1016/j.jenvman.2014.01.043.
30. Es-Soufi, R.; Tahiri, H.; Azaroual, L.; El Oualkadi, A.; Martin, P.; Badoc, A.; Lamarti, A. Biocontrol potential of Bacillus amyloliquefaciens Bc2 and Trichoderma harzianum TR against strawberry anthracnose under laboratory and field conditions. *Agricultural Sciences* **2020**, *11*, 260-277.
31. Fazion, F.; Perchat, S.; Buisson, C.; Vilas-Bôas, G.; Lereclus, D. A plasmid-borne Rap-Phr system regulates sporulation of Bacillus thuringiensis in insect larvae. *Environmental Microbiology* **2018**, *20*, 145-155, doi:https://doi.org/10.1111/1462-2920.13946.
32. Marín, A.; Ferreres, F.; Tomás-Barberán, F.A.; Gil, M.I. Characterization and Quantitation of Antioxidant Constituents of Sweet Pepper (Capsicum annuum L.). *Journal of Agricultural and Food Chemistry* **2004**, *52*, 3861-3869, doi:10.1021/jf0497915.
33. Webb, H.E.; Brichta-Harhay, D.M.; Brashears, M.M.; Nightingale, K.K.; Arthur, T.M.; Bosilevac, J.M.; Kalchayanand, N.; Schmidt, J.W.; Wang, R.; Granier, S.A.; et al. Salmonella in Peripheral Lymph Nodes of Healthy Cattle at Slaughter. *Frontiers in Microbiology* **2017**, *8*, doi:10.3389/fmicb.2017.02214.
34. Fan, B.; Chen, X.H.; Budiharjo, A.; Bleiss, W.; Vater, J.; Borriss, R. Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. *J. Biotechnol.* **2011**, *151*, 303-311.
35. Gao, T.; Wang, X.; Qin, Y.; Ren, Z.; Zhao, X. Watermelon Root Exudates Enhance Root Colonization of *Bacillus amyloliquefaciens* TR2. *Curr. Microbiol.* **2023**, *80*, doi:10.1007/s00284-023-03206-2.
36. Lu, X.; Liu, S.-F.; Yue, L.; Zhao, X.; Zhang, Y.-B.; Xie, Z.-K.; Wang, R.-Y. Epsc Involved in the Encoding of Exopolysaccharides Produced by *Bacillus amyloliquefaciens* FZB42 Act to Boost the Drought Tolerance of *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2018**, *19*, 3795, doi:10.3390/ijms19123795.
37. Constesini, F.J.; de Melo, R.R.; Sato, H.H. An overview of *Bacillus* proteases: from production to application. *Crit. Rev. Biotechnol.* **2018**, *38*, 321-334.
38. Gong, A.-D.; Li, H.-P.; Yuan, Q.-S.; Song, X.-S.; Yao, W.; He, W.-J.; Zhang, J.-B.; Liao, Y.-C. Antagonistic Mechanism of Iturin A and Plipastatin A from Bacillus amyloliquefaciens S76-3 from Wheat Spikes against Fusarium graminearum. *PLOS ONE* **2015**, *10*, e0116871, doi:10.1371/journal.pone.0116871.
39. Tian, Y.; Fan, Y.; Liu, J.; Zhao, X.; Chen, W. Effect of nitrogen, carbon sources and agitation speed on acetoin production of Bacillus subtilis SF4-3. *Electronic Journal of Biotechnology* **2016**, *19*, 41-49, doi:https://doi.org/10.1016/j.ejbt.2015.11.005.
40. Zhu, L.; Yang, X.; Xue, C.; Chen, Y.; Qu, L.; Lu, W. Enhanced rhamnolipids production by Pseudomonas aeruginosa based on a pH stage-controlled fed-batch fermentation process. *Bioresource Technology* **2012**, *117*, 208-213, doi:https://doi.org/10.1016/j.biortech.2012.04.091.
41. Ho, T.-H.; Chuang, C.-Y.; Zheng, J.-L.; Chen, H.-H.; Liang, Y.-S.; Huang, T.-P.; Lin, Y.-H. Bacillus amyloliquefaciens Strain PMB05 Intensifies Plant Immune Responses to Confer Resistance Against Bacterial Wilt of Tomato. *Phytopathology®* **2020**, *110*, 1877-1885, doi:10.1094/phyto-01-20-0026-r.
42. Chou, H.-P.; Huang, Y.-C.; Lin, Y.-H.; Deng, W.-L. Selection, Formulation, and Field Evaluation of *Bacillus amyloliquefaciens* PMB01 for Its Application to Manage Tomato Bacterial Wilt Disease. *Agriculture* **2022**, *12*, 1714, doi:10.3390/agriculture12101714.
43. Medeot, D.B.; Fernandez, M.; Morales, G.M.; Jofré, E. Fengycins From *Bacillus amyloliquefaciens* MEP218 Exhibit Antibacterial Activity by Producing Alterations on the Cell Surface of the Pathogens *Xanthomonas axonopodis* pv. *vesicatoria* and *Pseudomonas aeruginosa* PA01. *Front. Microbiol.* **2020**, *10*, 03107, doi:https://doi.org/10.3389/fmicb.2019.03107.
44. Wu, L.; Wu, H.; Chen, L.; Yu, X.; Borriss, R.; Gao, X. Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against Xanthomonas oryzae rice pathogens. *Sci. Rep.* **2015**, *5*, 12975, doi:10.1038/srep12975.
45. Bibi, S.; Weis, K.; Kaur, A.; Bhandari, R.; Goss, E.M.; Jones, J.B.; Potnis, N. A Brief Evaluation of Copper Resistance Mobile Genetic Island in the Bacterial Leaf Spot Pathogen, *Xanthomonas euvesicatoria* pv. *perforans*. *Phytopathology* **2023**, doi: https://doi.org/10.1094/PHYTO-02-23-0077-SC.
46. Obradovic, A.; Jones, J.B.; Momol, M.T.; Olson, S.M.; Jackson, L.E.; Balogh, B.; Guven, K.; Iriarte, F.B. Integration of Biological Control Agents and Systemic Acquired Resistance Inducers Against Bacterial Spot on Tomato. *Plant Dis.* **2005**, *89*, 712-716, doi:10.1094/pd-89-0712.
47. Kuriyama, I.; Musumi, K.; Yonezawa, Y.; Takemura, M.; Maeda, N.; Iijima, H.; Hada, T.; Yoshida, H.; Mizushina, Y. Inhibitory effects of glycolipids fraction from spinach on mammalian DNA polymerase activity and human cancer cell proliferation. *The Journal of Nutritional Biochemistry* **2005**, *16*, 594-601, doi:https://doi.org/10.1016/j.jnutbio.2005.02.007.