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Seed Treatment with Calcium Carbonate Containing *Bacillus amyloliquefaciens* PMB05 Powder Is an Efficient Way to Control Black Rot Disease of Cabbage

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Abstract: Black rot disease is a serious bacterial disease that harms vegetable crops of the *Brassica* genus (especially cabbage plants) worldwide. The causal agent, *Xanthomonas campestris* pv. *campestris* (Xcc), is a seed-borne pathogen that primarily infects seedlings. Previous studies suggest that the bacterial strain, *Bacillus amyloliquefaciens* PMB05, can intensify the plant immune responses of cabbage against black rot disease and reduce disease occurrence. In plant immunity, several reactions occur during a pathogen attack, but the elevation of calcium ion concentration in plant cells is essential in the induction of plant defense responses. Therefore, this study aims to investigate whether disease control of black rot disease in cabbage plants can be improved by integrating calcium carbonate in the formulation for preparing *B. amyloliquefaciens* PMB05. Firstly, we found the addition of calcium carbonate in the formulation revealed to have significantly increased the cell and endospore populations of *B. amyloliquefaciens* PMB05 in the fermentation liquids. To increase the convenience of disease control in the field, these fermentation liquids were converted to powder form for subsequent analysis. Results revealed that the grown seedlings from seeds, mixed with PMB05 powders, significantly intensified plant immune responses and improved black rot disease control. We further compared distinct seed treatments using one PMB05 powder to evaluate its feasibility in field application. The results demonstrated that the disease control efficacy and yield of cabbage were significantly improved in the seed treatment with the powder (SD-160C2) to 56.46% and 5.91%, respectively, at 10 weeks post transplanting. Interestingly, the seed treatment combined with a calcium-containing commercial fertilizer spraying treatment did not increase the control efficacy of black rot disease, but it significantly increased the weight of cabbages after harvest. We concluded that the seed treatment with calcium carbonate-containing *Bacillus amyloliquefaciens* PMB05 powder is an efficient way to control black rot disease in cabbage.

Keywords: biological control; field trial; plant immunity; powder; seed treatment

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1. Introduction

Cabbage (*Brassica oleracea*) is one of the vegetable crops with more than 8000 hectares planting area in Taiwan [1]. In the growing stage of cabbage, black rot disease caused by *Xanthomonas campestris* pv. *campestris* (Xcc) often occurs in environments with high humidity and temperature. This pathogen is mainly transmitted by seeds. It further invades the plant through its water holes, stomata, roots and wounds on the leaf edge and causes the typical symptoms of V-type gangrene on the leaf edge during plant growth. If the plant is left untreated, symptoms may lead to leaf drop. Black rot disease is considered one of the most destructive diseases of cruciferous plants [2–4]. To control these diseases, copper-containing fungicides and antibiotics are usually used in the field [5]. However, under long-term fungicide and antibiotic treatment application, drug-resistant bacteria strains have

gradually emerged [6–8]. Alternative control methods may include soaking seeds in hot water, but not only does this lead to a decrease in the germination rate of cabbage seeds, but it also fails to effectively eliminate pathogenic bacteria [9,10]. Therefore, the application of antagonistic microorganisms is considered to be an effective and environmentally friendly alternative in field application [11]. Many microorganisms that can regulate plant defense responses have also been reported to control the occurrence of various diseases [12–15]. In recent years, *Bacillus* spp. strains with antagonistic activity have been proven to inhibit the growth of Xcc and effectively reduce the occurrence of black rot disease [16–19]. In addition, our previous research demonstrated that the application of *Bacillus amyloliquefaciens* PMB05 bacterial suspension or powder on Xcc-contaminated cabbage seeds was able to reduce the occurrence of black rot disease in greenhouse assay [1,20]. The ability of PMB05 to supplement plant disease resistance is in regard to its ability to intensify plant immune responses of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [1,21–25].

PAMP-triggered immunity is the first layer of a plant's defense system during a pathogen attack. This defense response is initiated by pattern recognition receptors (PRRs) on the plant cell membrane after recognizing the PAMP of the pathogen, which activates the cellular signal transduction to enhance plant disease resistance [22,26–29]. PAMPs are generally conserved and unique structures of microorganisms. For example, flagellin is a PAMP from bacterial pathogens or chitin from fungal pathogens [30–32]. When PAMPs induce PTI, extracellular calcium influx will occur to activate NADPH oxidase to generate reactive oxygen species (ROS) [33–37]. ROS is not only an important indicator in the PTI pathway, but it can also participate in the regulation of mitogen-activated protein kinase (MAPK) activity. It then further activates defense signals such as salicylic acid (SA) or jasmonate ethylene (JA)/ethylene (ET) to cause plants to exhibit disease resistance [28,38–41]. *B. amyloliquefaciens* PMB05 intensifies PTI response in this manner [21]. Therefore, calcium plays a crucial role in the initial signal transduction of the plant defense response in the plant immune system. Integrating a calcium component in PMB05 formulations may increase the effect of PMB05 on PTI signal intensification in plants to attain improved disease control potential.

In this study, we first used calcium channel blockers to confirm that the function of *B. amyloliquefaciens* PMB05 in intensifying plant immune signals in cabbage leaves does require the participation of calcium. Subsequently, calcium carbonate, which is a material commonly used in the field as a calcium fertilizer, was used as the calcium source of the most efficient formulation in plant immunity intensification and control of black rot disease. Our previous study showed that the use of PMB05 powder on Xcc-contaminated seeds still maintains excellent disease control efficacy [1], and this application method is convenient for field application. We used the fermented liquid of PMB05 to make powders for subsequent analysis. After confirming that the grown seedling obtained from seed treatment with PMB05 powder is efficient in intensifying plant immune response and controlling black rot disease in cabbage plants in the greenhouse, the actual feasibility in the field becomes very important. We thus used the treatment of powder-treated seeds as a basis to evaluate the difference in disease control and yield in the field. The feasibility of the above treatment with a commercial calcium-containing product, Meili Calcium, was also evaluated.

In this study, we provided evidence to support that seed treatment with calcium carbonate-containing *B. amyloliquefaciens* PMB05 powder is efficient to control black rot disease in cabbage.

2. Materials and Methods

2.1. Growth Conditions for Cabbage Plants

The cabbage plant used in this study was *Brassica oleracea* L. var. *capitata*. Before assay, the seeds (Minghua Seed Co., New Taipei City, Taiwan) were sterilized with 1% sodium hypochlorite for 5 min and then washed three times with sterile water for 5 min each time. Afterwards, the seeds were regarded as clean seeds for use in further experiments. The seeds were sown individually in pots containing soil composed of vermiculite, pearlite and peat moss at a ratio of 10:1:1 ($v/v/v$). For plant immune response assays, the plants

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. For disease evaluation, plants were grown in the greenhouse at around 30 °C.

2.2. Growth Conditions of Bacterial Strains

The bacterial strain, *Xanthomonas campestris* pv. *campestris* XCC15, was cultured on a nutrient agar (NA) plate and incubated at 28 °C for 2 days. This was used as the source of inoculum. The bacterial suspension was prepared from colonies grown on the NA plates by washing them with sterilized distilled water containing 0.1% of CMC (Carboxymethylcellulose Sodium; Sigma, St. Louis, MO, USA). The OD_{600} value was adjusted to 0.3 (around 3×10^8 CFU/mL). Similarly, *Bacillus amyloliquefaciens* PMB05 was cultured on NA plates and incubated at 28 °C for 2 days. This was the source of the beneficial bacterium. To prepare the bacterial suspension of PMB05, one colony of PMB05 from the NA plate was inoculated in 5 mL of nutrient broth and incubated at 37 °C under 200 rpm for 16 h. Then, the bacteria culture was centrifuged at 8000 rpm for 5 min and further resuspended with sterilized water containing 0.1 % of CMC to 0.3 of OD_{600}.

2.3. Effect of Calcium Influx on Bacillus amyloliquefaciens PMB05-Intensifying Plant Immune Signals

To test if calcium is required for *B. amyloliquefaciens* PMB05 to intensify plant immune signals in cabbage leaves, the effect of Lanthanum chloride ($LaCl_3$, Sigma, Sigma, St. Louis, MO, USA) on ROS generation and callose deposition intensified by PMB05 were assayed upon treatment with the synthesized $flg22_{Xcc}$. The $flg22_{Xcc}$ (QRLSSGLRINSAKDDAAGLAIS) derived from *X. campestris* pv. *campestris* B305 (GenBank accession number DQ356465) was purchased from Life Tein LCC (South Plainfield, NJ, USA). In the assay, the final concentration of $flg22_{Xcc}$ at 1 μM was applied to the bacterial suspensions of PMB05. Then, the stock solution of $LaCl_3$ was added immediately to reach a final concentration of 40 μM and 80 μM. To evaluate the intensification of plant immune responses, rapid ROS generation and callose deposition were used as indicators and assayed according to the modified method [21]. The leaves of 4-week-old cabbage seedlings were infiltrated with each test solution, and then rapid ROS generation and callose deposition on leaf strips were evaluated at 1 h and 8 h post infiltration, respectively. In addition, in the follow-up experiment of powder application, $flg22_{Xcc}$ was directly infiltrated into the leaves of seedlings grown from powder-treated seeds for analysis. The evaluation of rapid ROS generation was performed by staining leaf strips with 20 μM of H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) (Molecular Probes, Eugene, OR, USA) in the dark for 20 min. Callose deposition was evaluated by staining leaf strips with 0.01% analine blue (Sigma, USA) in the dark for 2 h. The fluorescent results of rapid ROS generation and callose deposition were observed under a fluorescent microscope with the Excitation/Emission filter set at 465–495 nm/515–555 nm and 340–380 nm/400–425 nm, respectively. All of the images were taken under consistent conditions, and the ImageJ software (https://imagej.nih.gov/ij/, 1 March 2023) [21,22] was used to calculate the fluorescence intensities from each evaluation. In each experiment, 10 images were taken from each treatment as repeats, and three independent experiments were performed in this assay.

2.4. Effects of Calcium Influx on Bacillus amyloliquefaciens PMB05 in the Control of Black Rot Disease in Cabbage

To confirm that calcium influx is required for *B. amyloliquefaciens* PMB05 in the control of cabbage black rot, treatments with different final concentrations of $LaCl_3$ were used for a biocontrol assay. The bacterial suspensions of *X. campestris* pv. *campestris* XCC15 and *B. amyloliquefaciens* PMB05 were prepared by using 0.1% CMC in a ratio of 1:1 (v/v) mixed, and then $LaCl_3$ was added to obtain final concentrations of 0 μM, 40 μM and 80 μM in the mixtures. The leaves of the 4-week-old seedlings, prepared from clean seeds, were cut and inoculated 0.5 cm from the leaf's edge using scissors that were pre-sterilized with 75% alcohol and soaked in each mixture. The inoculated plants were bagged and placed in a

28 °C growth chamber. The lesion length from the inoculated cutting site to the end of developed symptoms was measured after 7 days post-inoculation. Each treatment had five seedlings as repeats, and three independent experiments were performed in this assay.

2.5. Preparation of Xcc-Contaminated Seed and Disease Severity Assay

To ensure the incidence and prevalence of black rot disease, cabbage seeds were contaminated with Xcc for all disease assays. A total of 1.6 g of clean seeds were soaked in a 10 mL bacterial suspension of *X. campestris* pv. *campestris* XCC15 for 1 h. The treated seeds were collected after drying in an air dryer (DHR-20TW, Cuisinart, Stamford, CT, USA). These seeds were regarded as "Xcc-contaminated seeds" for further experiments. The seeds were sown and grown in the greenhouse. The disease symptoms on the true leaves of 4-week-old plants were observed to calculate disease incidence and disease severity [42]. Disease severity was calculated based on the disease index (0 is no disease symptoms; 1 is weak water-soaked or yellowing symptoms; 2 is obvious yellowing in a small area from leaf edge; 3 is a V-shaped gangrene with necrosis symptoms). Disease severity was calculated using the following formula: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3)/(3 \times N_{Total})] \times 100\%$, whereby N_0-N_3 represented the number of plants in each different disease index and N_{Total} represented the summation of plants from N_0 to N_3 (the total number of plants tested in this experiment). Thirty plants were assayed in each treatment as repeats in a single experiment, and three independent experiments were performed in this assay.

2.6. Preparation of Bacillus amyloliquefaciens PMB05 Powder

To evaluate whether the addition of calcium carbonate to the formulations affects the beneficial effects of PMB05, the fermentation liquid was prepared based on the standard formulation, PMBFL-2A [23], with different concentrations of calcium carbonate: 0 mM, 2 mM, 10 mM, and 20 mM. The fermentation liquids were performed in a 30 L fermenter tank (BTF-B30L, Biotop Process & Equipment Inc., Nantou County, Taiwan) at 37 °C under 120 rpm and 1.5 vvm. The fermentation process took 5 days, and after each distinct fermentation liquid was obtained. Additionally, 20% maltodextrin (w/v) was added into each fermentation liquid. The fermentation liquids were further prepared into powders (labeled SD-160, SD-160C2, SD-160C10, and SD-160C20) using a spray dryer (GB210-A, KOHSIEH, Taipei, Taiwan) under conditions of a fixed flow rate at an inlet temperature of 160 °C [1]. The bacteria populations in these powders were evaluated.

2.7. Biocontrol Efficacy Assay of PMB05-Powder in Greenhouse and Field Trial

Both greenhouse and field trials were performed to test the efficacy of the powder in controlling black rot disease. In the greenhouse trials, 1% of each powder was mixed with the Xcc-contaminated seeds before sowing. After 4 weeks, the disease severity of each treatment was calculated. In the field trials, the experiment was conducted in Pingtung County (22.646191, 120.603503) from 28 October 2022 to 4 January 2023. Before starting the field trials, 30-day-old seedlings obtained from powder (SD-160C2)-treated clean seeds were prepared. Similar sized seedlings were selected for field planting. In order to ensure the consistent occurrence of black rot disease in the field, the bacterial suspension of XCC15 was sprayed every two weeks after two weeks of field planting. The disease control efficacy of the powder treatment was compared with non-powder treatment (blank) in the field. In order to confirm whether the effect of powder treatment on disease control can be enhanced by the addition of a commercial calcium-containing fertilizer, 500-fold diluted Meili calcium (Diamond Nano Biochem, Taichung, Taiwan) was used for treatment once every two weeks from two weeks post-planting. Disease severity in the field trial was calculated at 6, 8 and 10 weeks after field planting based on the disease index (0, no disease symptoms; 1, 1–5% diseased area; 2, 5–25% diseased area; 3, 26–50% diseased area; 4, over 51% diseased area). Disease severity was calculated using the following formula: $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)/(4 \times N_{total})] \times 100\%$, where N_0–N_4 represented the number of plants in each different disease index and N_{Total} represented the summation of plants from N_0 to N_4.

Control efficacy = [1–(disease severity of treatment/disease severity of blank)] × 100%. In addition, cabbages were harvested 10 weeks after planting. Cabbage yield was assessed by weighing the cabbage after cutting it from the base. In the above assays, data collected from six plants in one field plot were regarded as one repeat, and five plots were repeated in each treatment.

2.8. Data Analysis

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

3. Result

3.1. Effect of Calcium Influx on Bacillus amyloliquefaciens PMB05-Intensifying Plant Immune Signals

To ascertain whether calcium influx is required for *B. amyloliquefaciens* PMB05 in the intensification of plant immune responses in cabbage, the calcium channel blocker, $LaCl_3$, was applied to assay its effects on rapid ROS generation and callose deposition. The results showed that the $flg22_{Xcc}$-induced ROS generation was strongly intensified by the presence of PMB05 in cabbage leaves (Figure 1A). In the assay with the subsequent addition of $LaCl_3$, ROS generation was significantly reduced. However, there were no significant differences between the two concentrations tested (Figure 1B). For callose deposition, the $flg22_{Xcc}$-induced callose signal was intensified by PMB05. More significantly, in subsequent assays with the addition of $LaCl_3$, both concentrations completely eliminated callose deposition in the presence of PMB05 (Figure 2).

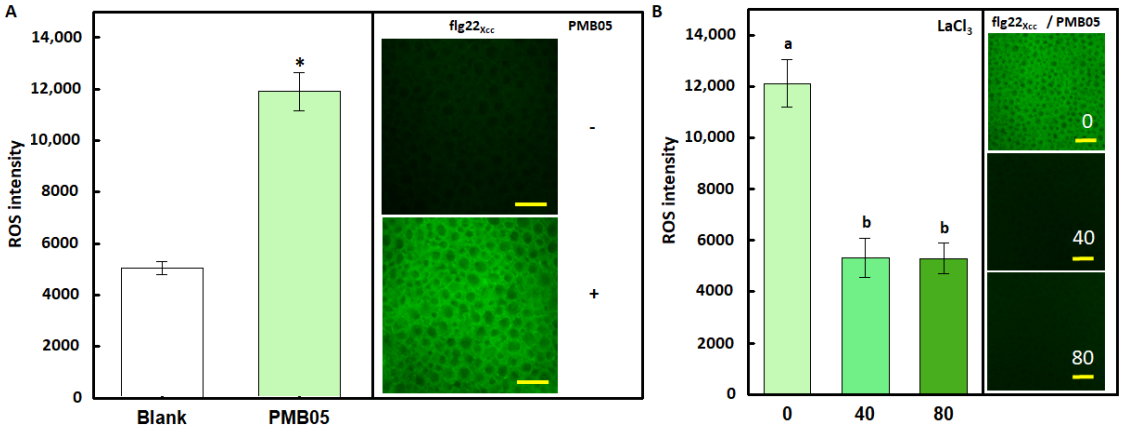

Figure 1. Effect of $LaCl_3$ on ROS generation intensified by *Bacillus amyloliquefaciens* PMB05 upon activation with $flg22_{Xcc}$. Panel (**A**) indicates the $flg22_{Xcc}$-induced ROS generation was intensified by *B. amyloliquefaciens* PMB05 on cabbage leaves. The symbols "+" and "−" indicate the application of distinct treatments for inclusion and exclusion of PMB05. The * above the columns indicates a significant difference compared to the blank treatment based on a *t* test ($p < 0.05$). Panel (**B**) indicates the *B. amyloliquefaciens* PMB05-intensified ROS generation was reduced by different concentrations of $LaCl_3$ (0, 40, 80 μM). Bar indicates 20 μm in length. Different letters (a, b) on the columns indicate significant differences between treatments based on Tukey's HSD ($F = 21.993$; $p < 0.05$).

3.2. Effects of Calcium Influx on Bacillus amyloliquefaciens PMB05 in Control of Black Rot Disease in Cabbage

To evaluate whether calcium influx is required in the control of black rot disease by *B. amyloliquefaciens* PMB05, the calcium channel blocker, $LaCl_3$, was applied in the biocontrol assay. The results showed that the symptoms of black rot disease were reduced by PMB05 without the addition of $LaCl_3$ (Figure 3A), while in the PMB05 treatments with

40 μM and 80 μM of $LaCl_3$ the symptoms were increased to 11.00 mm and 11.05 mm, respectively. The seeds treated with $LaCl_3$ displayed severer symptoms than those treated without $LaCl_3$ (Figure 3B).

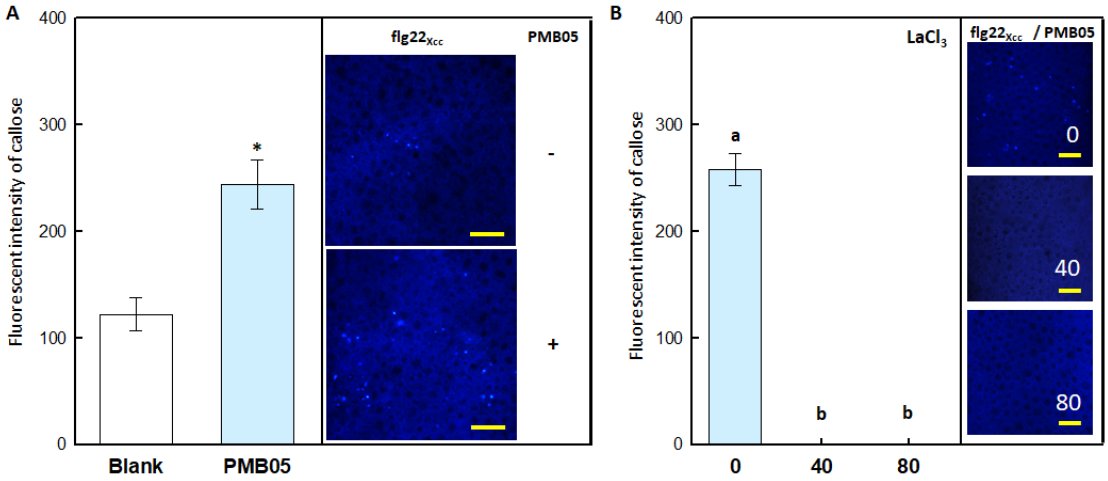

Figure 2. Effect of $LaCl_3$ on callose deposition intensified by *Bacillus amyloliquefaciens* PMB05 upon activation with $flg22_{Xcc}$. Panel (**A**) indicates the $flg22_{Xcc}$-induced callose deposition was intensified by *B. amyloliquefaciens* PMB05 on cabbage leaves. The symbols "+" and "−" indicate the application of distinct treatments for inclusion and exclusion of PMB05. The * above the columns indicates a significant difference compared to the blank treatment based on a *t* test ($p < 0.05$). Panel (**B**) indicates the *B. amyloliquefaciens* PMB05-intensified callose deposition was reduced by different concentrations of $LaCl_3$ (0, 40, 80 μM). Bar indicates 20 μm in length. Different letters (a, b) on the columns indicate significant differences between treatments based on Tukey's HSD ($F = 27.439$; $p < 0.05$).

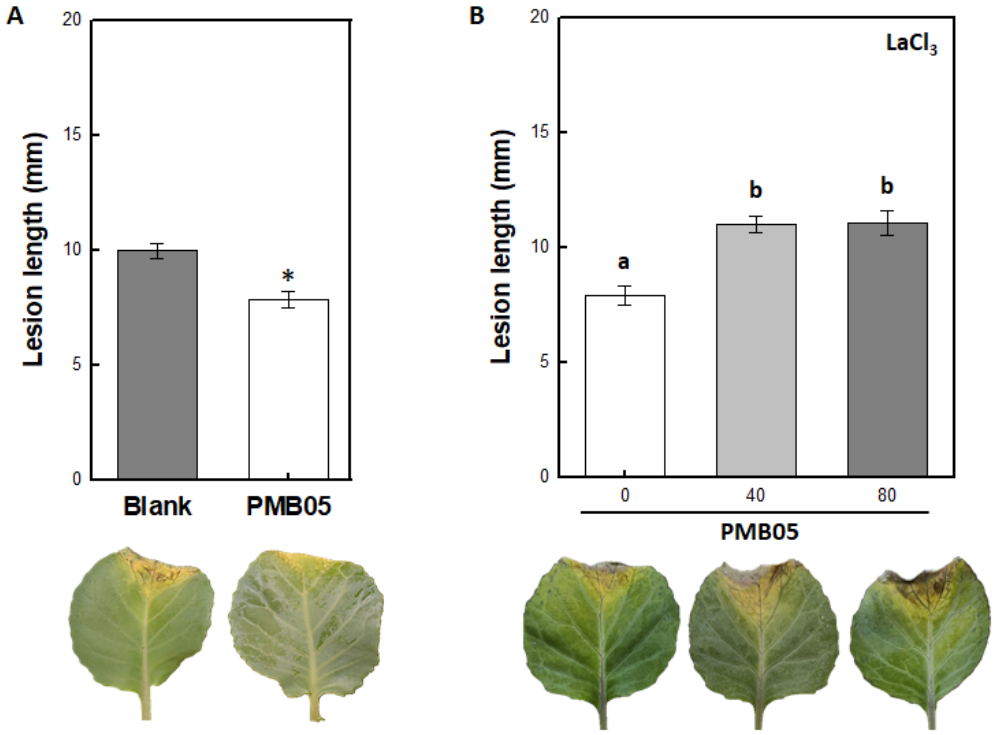

Figure 3. Effect of $LaCl_3$ on black rot disease controlled by *Bacillus amyloliquefaciens* PMB05. Panel (**A**) indicates the lesion length reduced by the treatment with bacterial suspension of *B. anyloliquefaciens* PMB05. The * above the columns indicates a significant difference compared to the blank treatment based on a *t* test ($p < 0.05$). Panel (**B**) indicates the effect of $LaCl_3$ on bacterial suspension of

B. anyloliquefaciens PMB05 in reducing lesion length of black rot disease. The seedlings were prepared from clean seeds as test materials. Before inoculation, the mixture of bacterial suspensions of *B. amyloliquefaciens* PMB05 and *Xanthomonas campestris* pv. *campestris* XCC15 was prepared by mixing them with equal volume. Then, the $LaCl_3$ was applied to make the final concentrations at 40 mM and 80 mM. The inoculation was carried out by cutting the leaves at 0.5 cm from the edge of the leaf with a scissor dipping in the mixture. Data were collected at 7 days post-inoculation. Different letters (a, b) on the columns indicated significant differences between treatment based on Tukey's HSD test ($F = 16.571$; $p < 0.05$).

3.3. Effects of Calcium Carbonate Application in the Preparation of the Bacillus amyloliquefaciens PMB05 Powder

To understand whether the addition of calcium carbonate in the formulations has an impact on the process of preparing the fermentation liquid of PMB05, the concentration of *B. amyloliquefaciens* PMB05 cells and endospores were evaluated. The results showed that the addition of calcium carbonate in formulations (PMBFL-2A2, PMBFL-2A10 and PMBFL-2A20) could not only increase the bacterial population of PMB05 in the fermentation liquid, but it could also increase the population of endospores (Table 1). However, there were no significant differences in the population of endospores among all formulations containing calcium carbonate. After using the aforementioned PMB05-fermentation liquids to prepare powders, SD-160C2, SD-160C10 and SD-160C20 containing calcium carbonate and SD-160 without calcium carbonate could be obtained. Further evaluation of the bacterial populations in these powders showed the bacterial populations in SD-160C2, SD-160C10 and SD-160C20 powders were about 9.92–10.10 (Log CFU/g), which were significantly higher than that in SD-160 powder (9.41 (Log CFU/g)).

Table 1. Evaluation of population of living cells and endospores of *B. amyloliquefaciens* PMB05 in formulations with different concentrations of calcium carbonate.

Powder		Fermentation Liquid		
Code	**Numbers (CFU/g)**	**Formulations**	**Numbers (CFU/mL)**	**Spores (CFU/mL)**
SD-160	9.41 ± 0.08^{b}	PMBFL-2A	8.59 ± 0.11^{b}	8.77 ± 0.17^{b}
SD-160C2	9.92 ± 0.18^{a}	PMBFL-2A2	9.44 ± 0.33^{a}	9.66 ± 0.21^{a}
SD-160C10	10.10 ± 0.04^{a}	PMBFL-2A10	9.40 ± 0.18^{a}	9.57 ± 0.10^{a}
SD-160C20	9.98 ± 0.05^{a}	PMBFL-2A20	9.57 ± 0.17^{a}	9.71 ± 0.02^{a}

Means of cell or endospore populations were presented in Log values. Different letters (a, b) indicate significant differences between treatments based on Tukey's HSD test (fermentation for cells and spores, $F = 12.7$ and 29.1, respectively; powder for cells, $F = 26.5$; $p < 0.05$).

3.4. Plant Immune Signals in Seedlings Grown from Powder-Treated Seeds

To evaluate the difference in the effects of intensifying plant immune signals on seedlings grown from seeds treated with different PMB05 powders, $flg22_{Xcc}$-induced rapid ROS and callose deposition on seedlings were analyzed. The results showed that both $flg22_{Xcc}$-induced ROS generation and callose deposition were intensified in the seed treatment with SD-160 (without calcium carbonate addition) in cabbage leaves. The above immune responses were further intensified in the seedlings from seed treatments with SD-160C2, SD-160C10 and SD-160C20. However, powders with more calcium carbonate in the formulation did not further intensify the plant immune signals (Figures 4 and 5).

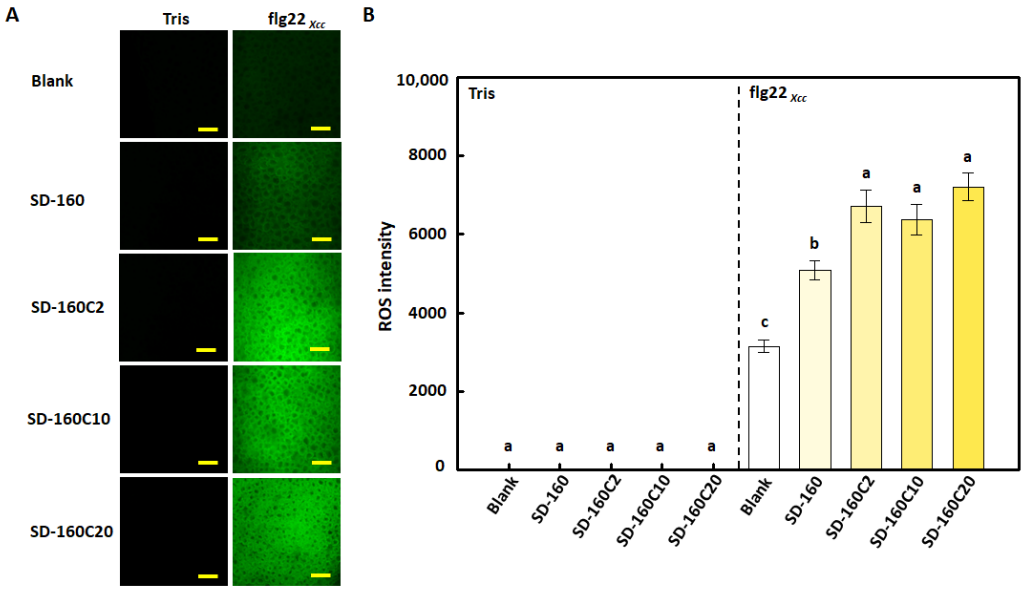

Figure 4. Effect of *Bacillus amyloliquefaciens* PMB05 in seedlings grown from different powder treatments on flg22$_{Xcc}$-induced ROS generation. The powder was obtained by spray-drying fermented liquid prepared from formulations containing different concentrations of calcium carbonate. In this assay, the seedlings grown after planting with clean seeds mixed with 1% powder were used as test materials. The leaves were infiltrated with flg22$_{Xcc}$ to trigger the ROS generation. Panel (**A**,**B**) indicate the fluorescent images and quantification of ROS intensity, respectively. Bar indicates 20 μm in length. Different letters (a, b, c) above the columns indicate significant differences between treatments based on Tukey's HSD test (In the flg22$_{Xcc}$ treatment, F = 40.328; $p < 0.05$).

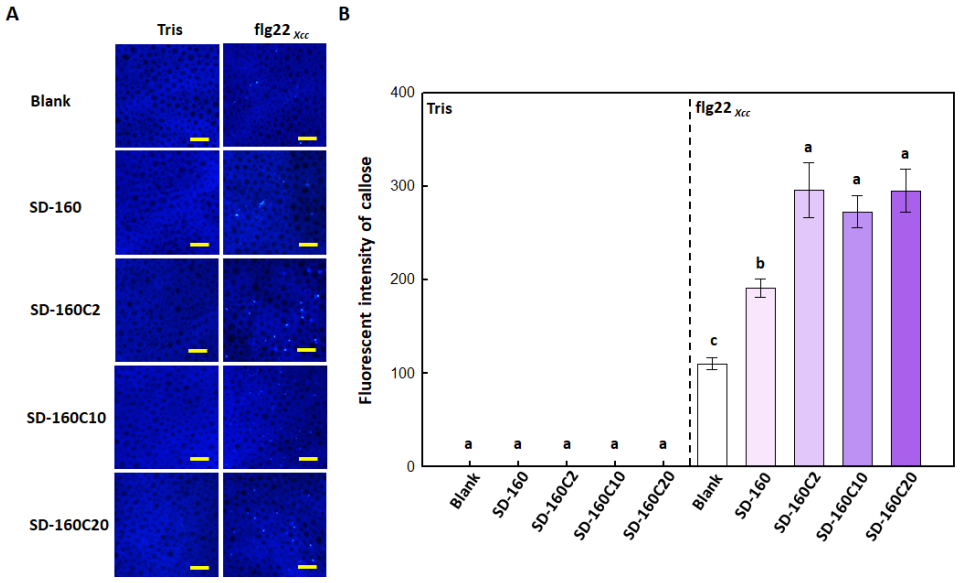

Figure 5. Effect of *Bacillus amyloliquefaciens* PMB05 in seedlings grown from different powder treatments on flg22$_{Xcc}$-induced callose deposition. The powder was obtained by spray-drying fermented liquid prepared from formulations containing different concentrations of calcium carbonate. In this assay, the seedlings grown after planting with clean seeds mixed with 1% powder were used as test materials. The leaves were infiltrated with flg22$_{Xcc}$ to trigger the callose deposition. Panel (**A**,**B**) indicate the fluorescent images and quantification of callose intensity, respectively. Bar indicates 20 μm in length. Different letters (a, b, c) above the columns indicate significant differences between treatments based on Tukey's HSD test (In the flg22$_{Xcc}$ treatment, F = 21.116; $p < 0.05$).

3.5. Biocontrol Assay of Seedlings Grown from Powder-Treated Seeds

To determine whether the occurrence of black rot disease on cabbage can be reduced by powder treatment on Xcc-contaminated seeds, disease incidence and disease severity were analyzed. The results revealed that all PMB05 powder-treated seeds significantly reduced the incidence of black rot disease after germination compared to that with untreated seeds (74.5%). In the seed treatment with SD-160, disease incidence was reduced to 42.2%. In addition, seeds treated with calcium carbonate-containing SD-160C2, SD-160C10 and SD-160C20 powder significantly reduced the incidence of black rot disease to 24.0%, 22.6%, and 22.0%, respectively (Figure 6A). The results were similar in terms of disease severity, and all powder treatment significantly reduced the severity of black rot disease. Among them, the treatment containing calcium carbonate powder was more effective in reducing the severity of disease symptoms than that without calcium carbonate (Figure 6B,C). However, there were no significant differences between the calcium carbonate-containing powder treatments in terms of disease incidence or disease severity.

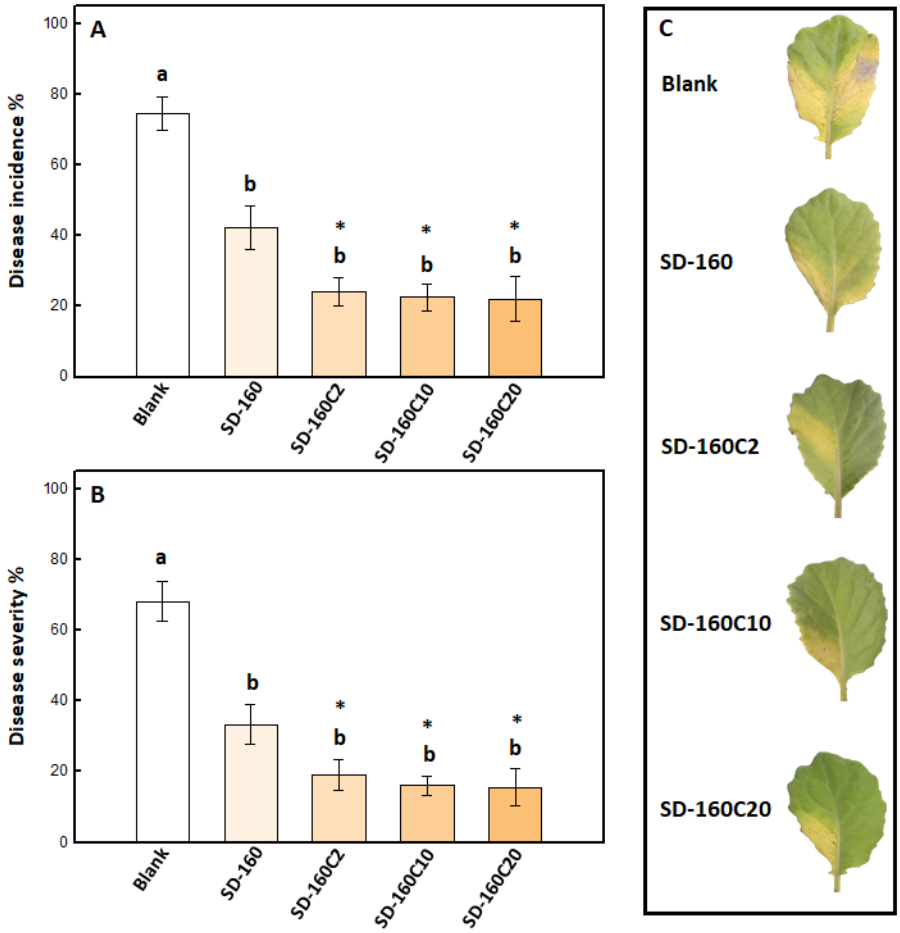

Figure 6. Effect of *Bacillus amyloliquefaciens* PMB05 in seedling grown from different powder treatments on the control of black rot disease. This experiment was conducted with 1% of PMB05-powders on Xcc-contaminated seeds. Panel (**A**,**B**) indicate the disease incidence and disease severity at 4 weeks after sowing, respectively. Different letters (a, b) above the columns indicate significant differences between treatments based on Tukey's HSD test (for disease incidence, F = 19.951; for disease severity, F = 22.872; $p < 0.05$). The * indicate a significant difference compared with the blank treatment, as assessed using a *t*-test ($p < 0.05$). Panel (**C**) exhibits the development of symptoms.

3.6. Effects of PMB05-Powder Seed Treatment on Disease Control and Yield of Cabbage in Field

To further confirm the actual situation of disease control in the field after the seeds were treated with PMB05 powder, we analyzed the occurrence of black rot disease and its

impact on cabbage yield under field trial. In terms of disease control, the results exhibited that in the first 8 weeks post-planting (WPP), the occurrence of black rot disease in the field was less (Figure 7A), and there was no significant difference among the treatments. The results after 10 WPP showed that the disease severity of the untreated blank treatment was significantly higher than that of the powder treatment (P). Meanwhile, the control efficacy of the PMB05 powder treatment was 56.5% (Figure 7B). In addition, the additional application of a commercial calcium-containing product (Meili Calcium) could still maintain the control effect of PMB05 powder, but there was no significant additive effect, and the control efficacy was 63.7%. In terms of yield, although the appearance of harvested cabbage in the PMB05 powder treatment (P) was slightly larger than that of the blank treatment (Figure 8A), there was no significant difference in weight (Figure 8B). Interestingly, in the additional treatment with Meili Calcium (PM), the harvested cabbage not only exhibited larger in appearance, but the weight of the harvested cabbage was significantly increased (by 51.6%).

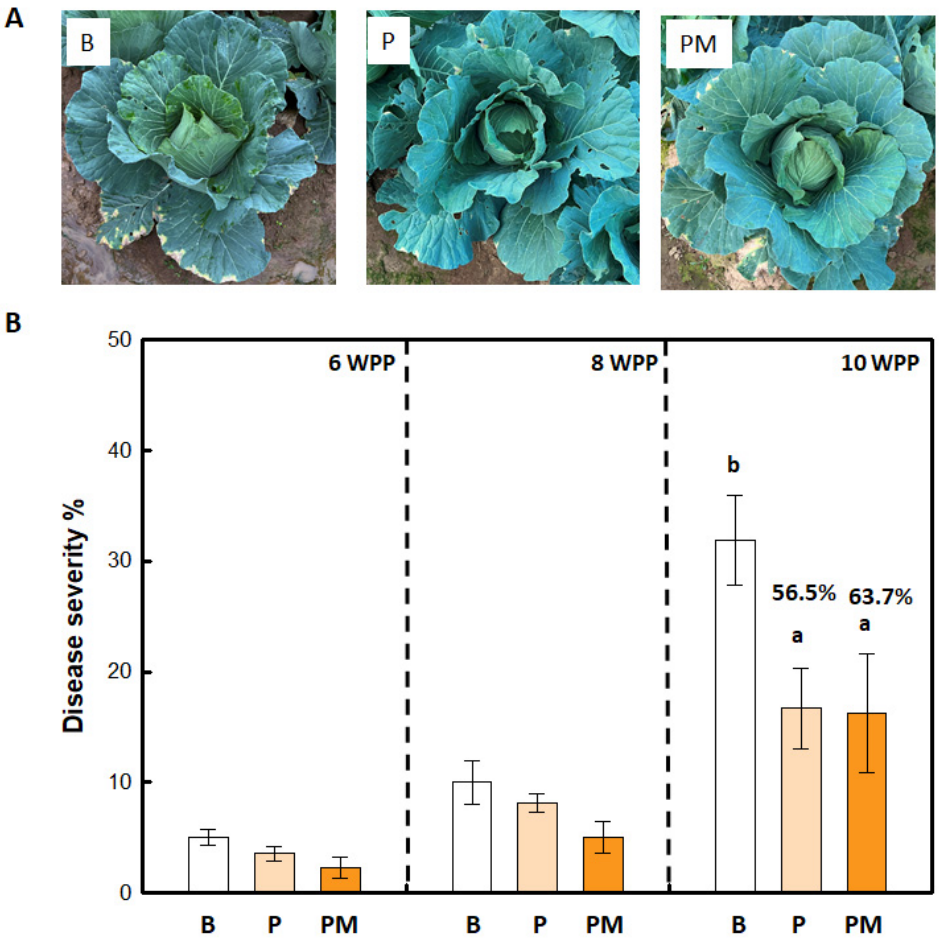

Figure 7. Effect of PMB05-powder on the control of cabbage black rot disease in the field. This experiment was conducted in a field in Neipu, Pingtung County in 2022. Panel (**A**) shows the symptom appearance of black rot disease at 6-week-post-planting (WPP). Panel (**B**) shows the disease severity of black rot disease at 6-, 8-, and 10-WPP. The powder treatment (P) was carried out with clean seeds mixed 1% of PMB05 powder (SD160-C2) before sowing for seedlings preparation. After the seedlings were planted in the field, bacterial suspensions of *Xanthomonas campestris* pv. *campestris* XCC15 were sprayed every two weeks to allow disease to occur. The results were collected at 6-, 8-, and 10-WPP. B indicates the untreated treatment, and PM indicates the powder treatment with additional spray of 500-fold dilution of Meili Calcium every two weeks. The numbers on the columns indicated the control efficacy. Different letters (a, b) above the columns indicate significant differences between treatments based on Tukey's HSD test ($F = 12.543$; $p < 0.05$).

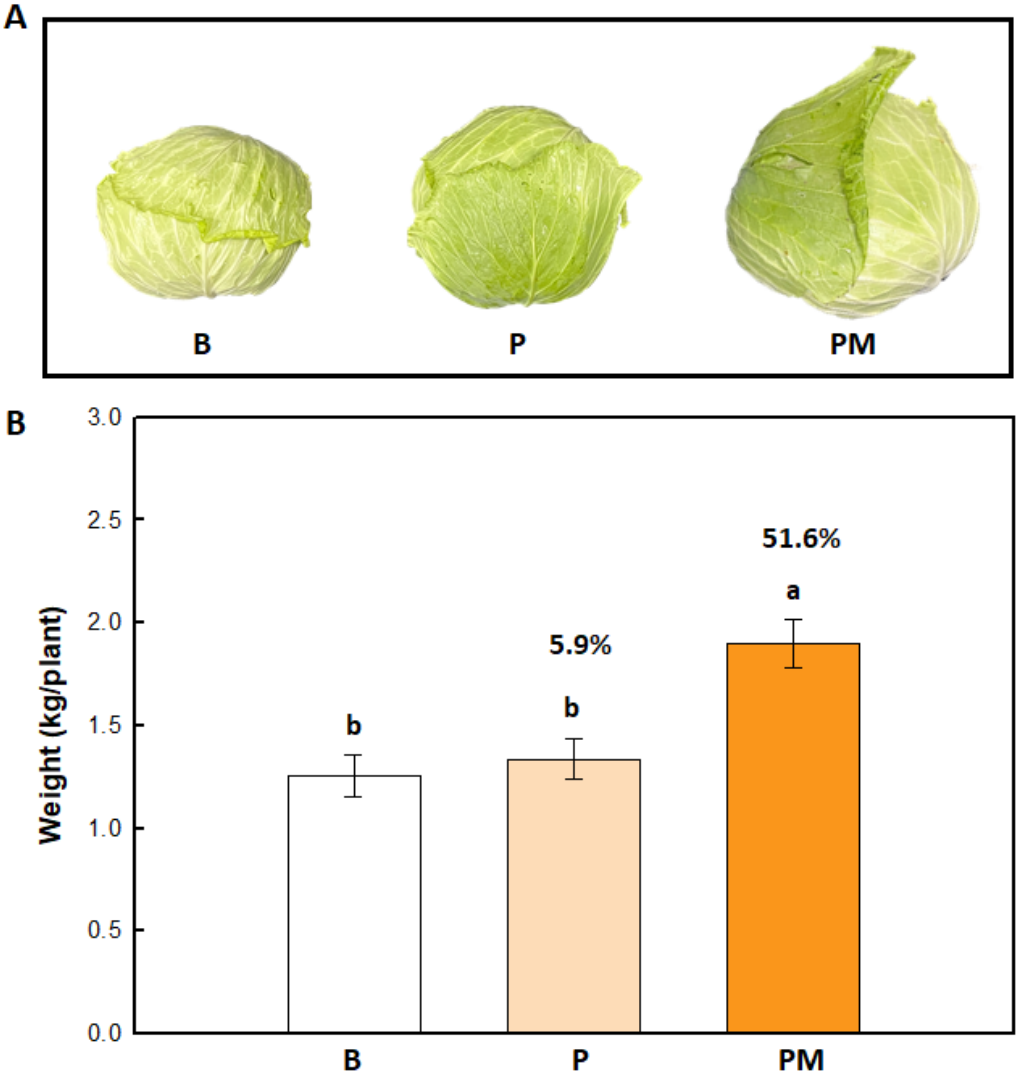

Figure 8. Effect of PMB05-powder on appearance and weight of harvested cabbage in the field trial. Panel (**A**) shows the harvested cabbage at 10-week-post-planting (WPP). Panel (**B**) shows the cabbage yield was assessed by weighing the cabbage after cutting it from the base. B indicates the untreated treatment, P indicates the powder treatment, and PM indicates the powder treatment with additional spray of 500-fold dilution of Meili Calcium every two weeks. In these assays, six plants in one field plot were regarded as one repeat, and five plots were repeated in each treatment. Different letters (a, b) above the columns indicate significant differences between treatments based on Tukey's HSD test ($F = 12.776$; $p < 0.05$).

4. Discussion

Black rot disease in cabbage caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is a common limiting factor in cabbage production [3,4]. Presently, the use of chemical pesticides, such as copper and antibiotics, are the major control methods [5]. However, long-term and extensive application of chemical agents will gradually produce drug-resistant strains [6–8]. Since black rot disease is a seed-borne disease, seed treatment technology is also a strategy that can be considered. Currently, hot water treatment is the most common method, but it often causes a decrease in germination rates, which can be regarded as an indirect loss [9,10]. In our previous studies, it was shown that the use of *Bacillus amyloliquefaciens* PMB05, whether the seed was soaked with fermentation liquid or mixed with bacterial powder, can effectively reduce the occurrence of black rot disease in laboratory analysis [1,20]. Thus, the use of biological control to control black rot disease

would be feasible in the field, and the improvement of its efficiency on disease control and its availability in the field is worth exploring. *B. amyloliquefaciens* PMB05 has been proven to enhance the PTI immune response of plants [21–24]; however, its effects on cabbage have yet to be investigated. During the activation of plant immune responses, calcium influx plays an important role for the further activation of plant defense pathways [40,43,44]. However, this process can be inhibited with a specific blocker, Lanthanum chloride ($LaCl_3$), to terminate further immune responses [45]. In this study, we provided evidence that the PTI immune response triggered by $flg22_{Xcc}$ was intensified by *B. amyloliquefaciens* PMB05 and that this intensification could be reduced by a calcium channel blocker, $LaCl_3$. We also found that the decrease in PTI immune signals caused by $LaCl_3$ further reduced the ability of *B. amyloliquefaciens* PMB05 to control black rot disease. Therefore, it is speculated that the ability of *B. amyloliquefaciens* PMB05 to control black rot disease on cabbage is indeed related to its intensified PTI and requires the involvement of calcium. It has also been reported that treatment of peppers with calcium-containing compounds (Maxifod Ca) induces plant defense responses and further increases resistance to *Fusarium* wilt disease [46]. Based on such findings, we believe that the addition of calcium to the fermented formulation of PMB05 may increase the control of black rot disease. Our preliminary study showed that the addition of calcium carbonate to the bacterial suspension of *B. amyloliquefaciens* PMB05 can effectively enhance plants' immune response and improve their ability to control tomato bacterial wilt. Therefore, we utilized the developed basal formulation, PMBFL-2A [23], to analyze its effect on the fermentation process after the addition of calcium carbonate. The results revealed that 2 mM of calcium carbonate in the formula was sufficient to increase the bacterial population in the fermentation liquid without affecting the production of endospores. However, this positive effect cannot be increased with an increase in the calcium carbonate concentration of the formulation. We have confirmed that these fermented liquids can be further prepared into powder form containing high bacterial populations. The obtained powders can be used to analyze their effects on $flg22_{Xcc}$-triggered plant immune signals and disease control.

To understand the relationship between the practical application of powder and plant immune response, we used seedlings grown from powder-treated seeds to analyze the immune response induced by $flg22_{Xcc}$. The results showed that powders containing calcium carbonate were more effective in intensifying plant immune signals than powders without calcium carbonate. However, higher concentrations of calcium carbonate did not intensify plant immune signals; there was no difference with lower concentrations of calcium carbonate. Therefore, we speculate that the addition of a low amount of calcium carbonate is sufficient for the *B. amyloliquefaciens* PMB05 in the powder to intensify plant immune signals. Interestingly, this intensified plant immune response in the leaves of seedling also suggests that *B. amyloliquefaciens* PMB05 may be endophytic in the tissues of cabbage. In the biocontrol assay carried out in the greenhouse, a similar trend was observed in the disease control effects of powder treatment on black rot disease with Xcc-contaminated seeds. The results suggested that treatment with PMB05 powder is indeed a simple way to control black rot disease. We observed that the strength of the immune response signal was positively correlated with disease resistance. However, we cannot rule out that this may have been due to the addition of calcium carbonate in the formulations or that it may have been only due to the increase in the bacterial populations of *B. amyloliquefaciens* PMB05. It is still unconfirmed if the addition of calcium carbonate to the formulations can effectively enhance plant immune response or increase resistance to black rot disease.

Since we demonstrated in the greenhouse assay that the PMB05 powder (SD-160C2) has a good disease control effect, it was important to evaluate if it can effectively reduce the occurrence of black rot disease under field trials. In the field trials, although the disease severity in the field was not high, the results demonstrated that the seed treatment with PMB05 powder could effectively reduce the occurrence of black rot disease in the field. An additional spray of Meili Calcium, applied every two weeks, did not reduce the disease

severity. This result is similar to the results of the previous greenhouse experiment. It can be speculated that the concentration of calcium ions in the plants planted in the field is sufficient for *B. amyloliquefaciens* PMB05 to achieve effective control. Additionally, the treatment of commercial Meili Calcium can effectively increase the weight of cabbage after harvest by more than 50%. The mechanism is still unknown and requires further investigation. It has been reported that cabbage seeds treated with hot water, along with subsequent integrated treatments, can reduce the occurrence of black rot disease in the field [47]. If this strategy is combined with the treatment developed in this study, it may significantly increase the reduction of cabbage black rot disease occurrence in the field. This is worth exploring for future research.

5. Conclusions

In this study, we conclude that the plant immune responses and disease resistance intensified by *Bacillus amyloliquefaciens* PMB05 can be enhanced by the addition of calcium carbonate to the fermentation formulations. The powder prepared from this formulation, and treating the PMB05 powder prepared according to this formulation on the seeds can effectively reduce the occurrence of black rot disease. Meanwhile, calcium carbonate-containing commercial products would not only affect the control efficacy of microorganisms to black rot disease, but also increase cabbage yield. Under the demand of reducing the use of fungicides in the world, the seed treatment of calcium carbonate-containing PMB05 powders can be recommended as an effective alternative way to control black rot disease in cabbage.

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References

1. Hsiao, C.-Y.; Yan, J.-C.; Peng, A.-L.; Chen, B.-W.; Li, C.-R.; Lin, Y.-H. Seed treatment of Bacillus amyloliquefaciens PMB05 powder for control black rot disease of cabbage. *J. Plant Med.* **2022**, *64*, 149–157. [CrossRef]
2. Russell, H.L. *A Bacterial Rot of Cabbage and Allied Plants*; University of Wisconsin, Agricultural Experiment Station: Arlington, WI, USA, 1898.
3. Alvarez, A.M. Black Rot of Crucifers. In *Mechanisms of Resistance to Plant Diseases*; Slusarenko, A.J., Fraser, R.S.S., van Loon, L.C., Eds.; Springer: Dordrecht, The Netherlands, 2000; pp. 21–52.
4. Vicente, J.G.; Holub, E.B. Xanthomonas campestris pv. campestris (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. *Mol. Plant Pathol.* **2013**, *14*, 2–18. [CrossRef] [PubMed]
5. Gupta, M.; Vikram, A.; Bharat, N. Black rot-A devastating disease of crucifers: A review. *Agric. Rev.* **2013**, *34*, 269–278. [CrossRef]
6. Voloudakis, A.E.; Bender, C.L.; Cooksey, D.A. Similarity between Copper Resistance Genes from *Xanthomonas campestris* and *Pseudomonas syringae*. *Appl. Environ. Microbiol.* **1993**, *59*, 1627–1634. [CrossRef] [PubMed]
7. Sundin, G.W.; Bender, C.L. Expression of the strA-strB streptomycin resistance genes in Pseudomonas syringae and Xanthomonas campestris and characterization of IS6100 in X. campestris. *Appl. Environ. Microbiol.* **1995**, *61*, 2891–2897. [CrossRef]
8. Hsiao, Y.-M.; Liu, Y.-F.; Lee, P.-Y.; Hsu, P.-C.; Tseng, S.-Y.; Pan, Y.-C. Functional Characterization of copA Gene Encoding Multicopper Oxidase in *Xanthomonas campestris* pv. *campestris*. *J. Agric. Food Chem.* **2011**, *59*, 9290–9302. [CrossRef]
9. Babadoost, M.; Derie, M.; Gabrielson, R. Efficacy of sodium hypochlorite treatments for control of *Xanthomonas campestris* pv. *campestris in Brassica seeds. Seed Sci. Technol.* **1996**, *24*, 7–15.

10. van der Wolf, J.M.; van der Zouwen, P.S.; van der Heijden, L. Flower infection of Brassica oleracea with Xanthomonas campestris pv. campestris results in high levels of seed infection. *Eur. J. Plant Pathol.* **2013**, *136*, 103–111. [CrossRef]
11. Okorski, A.; Oszako, T.; Nowakowska, J.A.; Pszczółkowska, A. The Possibilities of Biologically Protecting Plants against Diseases in Nurseries, with Special Consideration of Oomycetes and Fusarium Fungi. *For. Res. Pap.* **2014**, *75*, 301–321. [CrossRef]
12. Attia, M.S.; Abdelaziz, A.M.; Al-Askar, A.A.; Arishi, A.A.; Abdelhakim, A.M.; Hashem, A.H. Plant growth-promoting fungi as biocontrol tool against *Fusarium* wilt disease of tomato plant. *J. Fungi* **2022**, *8*, 775. [CrossRef]
13. Riera, N.; Wang, H.; Li, Y.; Li, J.; Plez-Stelinski, K.; Wang, N. Induced systemic resistance against citrus canker disease by rhizobacteria. *Phytopathology* **2018**, *108*, 1038–1045. [CrossRef] [PubMed]
14. Samaras, A.; Roumeliotis, E.; Ntasiou, P.; Karaoglanidis, G. *Bacillus subtilis* MBI600 promotes growth of tomato plants and induces systemic resistance contributing to the control of soilborne pathogens. *Plants* **2021**, *10*, 1113. [CrossRef]
15. Wu, G.; Liu, Y.; Xu, Y.; Zhang, G.; Shen, Q.; Zhang, R. Exploring elicitors of the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Mol. Plant-Microbe Interact.* **2018**, *31*, 560–567. [CrossRef]
16. Liu, K.; Garrett, C.; Fadamiro, H.; Kloepper, J.W. Antagonism of black rot in cabbage by mixtures of plant growth-promoting rhizobacteria (PGPR). *BioControl* **2016**, *61*, 605–613. [CrossRef]
17. Mácha, H.; Marešová, H.; Juříková, T.; Švecová, M.; Benada, O.; Škríba, A.; Baránek, M.; Novotný, Č.; Palyzová, A. Killing Effect of Bacillus velezensis FZB42 on a *Xanthomonas campestris* pv. *Campestris* (Xcc) Strain Newly Isolated from Cabbage *Brassica oleracea* Convar. *Capitata* (L.): A Metabolomic Study. *Microorganisms* **2021**, *9*, 1410.
18. Massomo, S.M.S.; Mortensen, C.N.; Mabagala, R.B.; Newman, M.-A.; Hockenhull, J. Biological Control of Black Rot (*Xanthomonas campestris* pv. *campestris*) of Cabbage in Tanzania with Bacillus strains. *J. Phytopathol.* **2004**, *152*, 98–105. [CrossRef]
19. Monteiro, L.; Mariano, R.d.L.R.; Souto-Maior, A.M. Antagonism of Bacillus spp. against *Xanthomonas campestris* pv. *campestris*. *Braz. Arch. Biol. Technol.* **2005**, *48*, 23–29. [CrossRef]
20. 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. [CrossRef]
21. 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**, *112*, 2495–2502. [CrossRef] [PubMed]
22. 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. [CrossRef]
23. 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.
24. Wang, Y.-H.; Lai, I.-L.; Zheng, J.-L.; Lin, Y.-H. Using Dynamic Changes of Chlorophyll Fluorescence in Arabidopsis thaliana to Evaluate Plant Immunity-Intensifying Bacillus spp. Strains. *Phytopathology®* **2019**, *109*, 1566–1576. [CrossRef]
25. 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. [CrossRef]
26. Dodds, P.N.; Rathjen, J.P. Plant immunity: Towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* **2010**, *11*, 539–548. [CrossRef]
27. Hetmann, A.; Kowalczyk, S. [Suppression of PAMP-triggered immunity (PTI) by effector proteins synthesized by phytopathogens and delivered into cells of infected plant]. *Postep. Biochem.* **2019**, *65*, 58–71. [CrossRef] [PubMed]
28. Jones, J.D.G.; Dangl, J.L. The plant immune system. *Nature* **2006**, *444*, 323–329. [CrossRef] [PubMed]
29. Postel, S.; Kemmerling, B. Plant systems for recognition of pathogen-associated molecular patterns. *Semin. Cell Dev. Biol.* **2009**, *20*, 1025–1031. [CrossRef]
30. Baureithel, K.; Felix, G.; Boller, T. Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitooligosaccharides and a Nod factor of Rhizobium. *J. Biol. Chem.* **1994**, *269*, 17931–17938. [CrossRef] [PubMed]
31. Felix, G.; Duran, J.D.; Volko, S.; Boller, T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **1999**, *18*, 265–276. [CrossRef]
32. Kunze, G.; Zipfel, C.; Robatzek, S.; Niehaus, K.; Boller, T.; Felix, G. The N Terminus of Bacterial Elongation Factor Tu Elicits Innate Immunity in Arabidopsis Plants. *Plant Cell* **2004**, *16*, 3496–3507. [CrossRef] [PubMed]
33. Kwaaitaal, M.; Huisman, R.; Maintz, J.; Reinstädler, A.; Panstruga, R. Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochem. J.* **2011**, *440*, 355–373. [CrossRef] [PubMed]
34. Ranf, S.; Eschen-Lippold, L.; Pecher, P.; Lee, J.; Scheel, D. Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* **2011**, *68*, 100–113. [CrossRef]
35. Kadota, Y.; Shirasu, K.; Zipfel, C. Regulation of the NADPH Oxidase RBOHD during Plant Immunity. *Plant Cell Physiol.* **2015**, *56*, 1472–1480. [CrossRef]

36. Ogasawara, Y.; Kaya, H.; Hiraoka, G.; Yumoto, F.; Kimura, S.; Kadota, Y.; Hishinuma, H.; Senzaki, E.; Yamagoe, S.; Nagata, K.; et al. Synergistic Activation of the Arabidopsis NADPH Oxidase AtrbohD by Ca^{2+} and Phosphorylation*. *J. Biol. Chem.* **2008**, *283*, 8885–8892. [CrossRef]
37. Qu, Y.; Yan, M.; Zhang, Q. Functional regulation of plant NADPH oxidase and its role in signaling. *Plant Signal Behav.* **2017**, *12*, e1356970. [CrossRef]
38. Camejo, D.; Guzmán-Cedeño, Á.; Moreno, A. Reactive oxygen species, essential molecules, during plant–pathogen interactions. *Plant Physiol. Biochem.* **2016**, *103*, 10–23. [CrossRef]
39. Luna, E.; Pastor, V.; Robert, J.; Flors, V.; Mauch-Mani, B.; Ton, J. Callose Deposition: A Multifaceted Plant Defense Response. *Mol. Plant-Microbe Interact.*® **2011**, *24*, 183–193. [CrossRef]
40. Torres, M.A. ROS in biotic interactions. *Physiol. Plant.* **2010**, *138*, 414–429. [CrossRef]
41. Wang, Y.; Li, X.; Fan, B.; Zhu, C.; Chen, Z. Regulation and Function of Defense-Related Callose Deposition in Plants. *Int. J. Mol. Sci.* **2021**, *22*, 2393. [CrossRef] [PubMed]
42. Winstead, N.N.; Kelman, A. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. *Phytopathology* **1952**, *42*, 628–634.
43. Dubiella, U.; Seybold, H.; Durian, G.; Komander, E.; Lassig, R.; Witte, C.-P.; Schulze, W.X.; Romeis, T. Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8744–8749. [CrossRef]
44. Scheler, C.; Durner, J.; Astier, J. Nitric oxide and reactive oxygen species in plant biotic interactions. *Curr. Opin. Plant Biol.* **2013**, *16*, 534–539. [CrossRef]
45. Su, Y.-H.; Hong, C.-Y.; Lin, Y.-H. Plant ferredoxin-like protein enhances resistance to bacterial soft rot disease through PAMP-triggered immunity in Arabidopsis thaliana. *Eur. J. Plant Pathol.* **2014**, *140*, 377–384. [CrossRef]
46. Attia, M.S.; Elsayed, S.M.; Abdelaziz, A.M.; Ali, M.M. Potential impacts of *Ascophyllum nodosum*, *Arthrospira platensis* extracts and calcium phosphite as therapeutic nutrients for enhancing immune response in pepper plant against *Fusarium* wilt disease. *Biomass Convers. Biorefinery* **2023**. [CrossRef]
47. Ombuna, G.; Nyangeri, B.; Maobe, S. Control of black rot disease in cabbage by integration of mulching, pruning and hot water treatment of seeds. *Plannt Pathol. Quar.* **2019**, *9*, 23–29. [CrossRef]