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RESEARCH ARTICLE

Bacillus amyloliquefaciens Activates the Basal Defense of Chinese Cabbage Against *Alternaria brassicicola*

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Abstract *Alternaria brassicicola* is a necrotrophic plant pathogen that causes substantial damage to a broad range of host plants, especially Brassica species. Various strategies were employed to control this disease, including the application of antagonistic microorganisms. In this study, we evaluate two strains of *Bacillus amyloliquefaciens* (PMB04 and PMB05) as biological control agents for black leaf spot disease caused by *A. brassicicola* on Chinese cabbage (*Brassica rapa* var. *chinensis*). We found that these two strains inhibited mycelial growth and spore germination of *A. brassicicola* and reduced disease severity on Chinese cabbage. However, PMB04 showed a better *in vitro* inhibitory effect than PMB05, but PMB05 has a better biocontrol efficacy than PMB04. To gain more insight into the relationship between the inhibitory effect of these two strains and their biocontrol efficacy, the plant immune response was enhanced with the *B. amyloliquefaciens* bacterial cell and its cultural filtrate. The results exhibited that both forms of PMB05 significantly intensify the plant immunity in leaf tissue upon the inoculation of *A. brassisicola* spores, rather than those of PMB04. This suggests that *B. amyloliquefaciens* strain PMB04 and PMB05 have different biocontrol mechanisms.

Keywords: Biocontrol mechanism, black leaf spot disease, callose deposition, swollen conidia.

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Introduction

Chinese cabbage (*Brassica rapa* var. *chinensis*) is a Brassicaceae species widely grown in several Asian nations. It is a commercially significant vegetable owing to its strong nutritional content and savory flavor. Abiotic and biotic stress, including climate change, and an increase in the number of disease races and variations, are all important issues that endanger this plant's production [1]. Several phytopathogens have been found to infect Brassicaceae species, such as soft rot pathogens [2], turnip mosaic virus [3], Downey mildew [4], as well as the fungus *Alternaria brassicicola* [5], which can infect the leaves of immature plants in nurseries, causing leaf spots and damping off.

Chemical fungicides and antagonistic microorganisms were widely used as control measures. Among some popular biocontrol agents, *Bacillus* spp. have been shown to efficiently suppress fungal phytopathogens because they generate a variety of antifungal compounds and have an ecologically benign behavior [6]. This genus has previously been shown to activate the PGPR-mediated induced systemic resistance (ISR) response, causing plants to produce defense compounds that have protective

effects against plant pathogens [7, 8]. There is a strong correlation between this acquired disease resistance and PAMP-triggered immunity (PTI), the plant's main defense mechanism [9][10].

Several investigations have shown that *B. amyloliquefaciens* is extremely effective, not just in lab-scale experiments. The effectiveness of *B. amyloliquefaciens* in controlling a variety of phytopathogens has been proven on anthracnose in mango fruit [11], soft rot of postharvest chili pepper [12], powdery mildew on tobacco [13], and fungal diseases on maize caused by *F. verticillioides, Rhizopus stolonifera*, and *Penicillium variabile* [14]. In ten field studies, *B. amyloliquefaciens* PMB01 decreased bacterial wilt severity in tomatoes by more than 67% [15]. Furthermore, seed treatment with calcium carbonate-containing *B. amyloliquefaciens* PMB05 powder is an effective method of controlling black rot disease in cabbage by intensifying the plant's immune response [16].

Moreover, investigations have shown that *B. amyloliquefaciens'* culture filtrate can suppress infection. Fermentation broth of *B. amyloliquefaciens* could effectively control the occurrence of bacterial leaf spot disease by using the 200- or 500-fold dilution [17]. Cultural filtrate or fermentation broth was obtained from the cell-free supernatant, which contains phytohormones, siderophores, proteins, peptides, organic acids, and volatile compounds [18]. Numerous investigations have demonstrated the widespread presence of lipopeptides in *Bacillus* sp. [19, 20]. The inhibitory activities of these compounds against different bacterial pathogens all play an important role in disease control.

The induced immune system could be analyzed from the callose deposition [21]. *B. amyloliquefaciens* strain PMB05 has been demonstrated to enhance flg22pst- or harpin-triggered PTI response, such as callose deposition [8]. Callose plays a vital role in plants' defense systems. Callose is formed between the plasma membrane and the cell wall at pathogen infection sites, plasmodesmata, and other plant tissues to limit disease penetration and distribution [22, 23]. More research was needed to determine if *B. amyloliquefaciens* suppressed *A. brassicicola in vitro*. Thus, this study investigated the immunological responses of *B. rapa* var. *chinensis* upon the application of *B. amyloliquefaciens* PMB04 and PMB05 culture filtrate and bacterial cells against *A. brassicicola*. This study addressed a knowledge gap about the biocontrol efficiency of *B. amyloliquefaciens* against particular phytopathogens, such *A. brassicicola,* which causes black spot disease in Chinese cabbage.

Materials and Methods

Microbial Strains and Cultural Conditions

Bacillus amyloliquefaciens strains PMB04 and PMB05 were utilized as biocontrol agent bacteria in this investigation, with *A. brassicicola* strain ALB1 serving as the pathogen. Phytobacteriology Laboratory, Department of Plant Medicine, National Pingtung University of Science and Technology, Taiwan, provided all microbial strains.

For bacterial cell culture, the pure culture of PMB04 and PMB05 was maintained in NA medium (Nutrient Agar 23 g/L) (Sigma Aldrich, USA) for 2-3 days. The suspension of bacteria PMB04 and PMB05 was prepared as follows: the bacteria were added to NB Medium (Nutrient Broth 8 g/L) and incubated for 48 hours at 29°C in an incubator shaker at 150 rpm. Bacteria suspension centrifuged at 8000 rpm, 4°C for 3-5 minutes. After centrifugation, the supernatant was discarded, and the pellet was washed and resuspended with ddH_2O and carboxymethylcellulose sodium (CMC) to obtain a final bacterial population density of OD600 0.3 or 10^8 CFU/mL using a spectrophotometer (CT 2800 Spectrophotometer, Taiwan). ddH2O was used for dual culture assay, while CMC suspension was used for treatment on plants, such as disease severity assay and callose deposition assay.

For the preparation of both *B. amyloliquefaciens* strains, culture filtrate was maintained on NB (Nutrient broth 8g/L) for 16 hours at 37°C shaker at 150 rpm, to obtain a bacterial population. The broth was centrifuged at 5500 rpm, 24°C for 3-5 minutes. After centrifugation, the supernatant is discarded and resuspended with ddH_2O to obtain a final OD_{600} = 0.3. And then, 1 mL of both *B. amyloliquefaciens* suspension was added to 10 mL of 523 Medium (10 g sucrose, 8 g casein dehydrolysate, 4 g yeast extract, 2 g KH_2PO_4, 0.3 g $MgSO_4$, pH 7.0), the medium was shaken at 150 rpm, 30°C for 8 hours. Then, PMB04 and PMB05 are centrifuged and prepared at a final OD600 = 0.3. One mL of the suspension was added to 100 mL SYM medium (20 g brown sugar, 10 g soy powder, 5 g yeast extract, 1.6 g K_2HPO_4, 0.8 g KH_2PO_4, 03 g $MgSO_4$) and shaken at 150 rpm, 30°C for 5 days. PMB04 and PMB05 suspension are centrifuged at 5500 rpm on 5°C for 10 minutes, then filtered using a 0.22 µm sterile syringe filter and stored in a 4°C fridge. As a pathogen strain, *A. brassicicola* (ALB1) was cultured in potato dextrose agar (PDA) at 28°C for 7 days.

Inhibition Test

Inhibitory assessment of *B. amyloliquefaciens* toward *A. brassicicola* was performed through the measurement of *A. brassicicola* mycelial growth on agar media and its spore germination. PMB04 and PMB05 were tested in combination against *A. brassisicola* using a dual culture technique. Bacterial suspension was prepared with sterile distilled water and adjusted to an OD_{600} = 0.3 (equivalent to 10^8 CFU/mL) using a spectrophotometer (CromTech, CT-2800, Taiwan). A mycelial disc (10 mm) from a 7-day-old culture of *A. brassisicola* was placed at the center of the NA-PDA plate for 36 hours. Then, each plastic ring that was placed around the A. brassisicola mycelial disk was dripped with 20 µL sterile distilled water, and PMB04 and PMB05 suspension (Figure 3A). Twelve replicates were used for each treatment, and the experiment was repeated five times. The plates were incubated at 28°C for 7 days, and the inhibition zone was measured using a Vernier caliper and the inhibitory rate was calculated as follows:

$$\text{Inhibitory rate (\%)} = \frac{\text{Inhibition of water} - \text{Inhibition of bacteria}}{\text{Inhibition of water}} \times 100\%$$

To assay the spore germination rate of *A. brassicicola* toward the application of *B. amyloliquefaciens*, ALB1 spore suspensions (100 µL, 10^3 conidia/mL) were prepared with 100 µL glucose in a 2 mL Eppendorf tube for blank treatment. The other treatments were spore suspension added with glucose and 100 µL each cultural filtrate of PMB04 and PMB05. Each treatment was placed on a strip well microplate (Costar, USA) and incubated at 28°C. After 12 h, the spore germination was observed and captured using LAS software. This experiment was performed with four replicates in each treatment. Germination and swelling rates are measured, and the germination percentage over the control was calculated. The data were analyzed based on Tukey's multiple range test ($p < 0.05$).

Biocontrol Efficacy

Evaluation of the biocontrol activity of *B. amyloliquefaciens* PMB04 and PMB05 toward black leaf spot caused by *A. brassicicola* was performed on 2-week-old true leaves of Chinese cabbage that were cultivated in a greenhouse. The PMB04 and PMB05 bacterial cell suspensions were prepared as described earlier. For the control experiment, the leaf was sprayed with sterilized water. Chinese cabbage leaves were sprayed with 1 mL of the cell suspension of PMB04 and PMB05, for the second and third treatments, respectively. After air drying the leaves, the center of each leaf was inoculated with 5 µL of *A. brassisicola* $4x10^6$ conidia/mL. Disease severity was scored after 7 days by evaluating the lesion of each leaf according to the following indices: 0 categorized as the healthy leaf; 1 categorized when there is a small black spot; 2 categorized when the color changes to brown; 3 categorized when the necrotic area is bigger than index number 2; 4 categorized when the leaf starts wilting. Means of disease severity were calculated from 50 samples in each of three independent experiments using the following formula:

$$\text{DS (\%)} = \frac{\sum(\text{nx0}) + (\text{nx1}) + (\text{nx2}) + (\text{nx3}) + (\text{nx4})}{\text{total n}} \times 100\%$$

Activation of Plant Immunity

Callose deposition assays are employed to evaluate the induced immune system of Chinese cabbage upon inoculation with PMB04 and PMB05 challenged with *A. brassiscicola*. Cultural filtrate and bacterial cell suspension of PMB04 and PMB05 were prepared. Both kinds of suspension are compared for further assay on the leaves. The stimulus-induced callose spot quantification technique was modified from 9, 10, 24. Chinese cabbage leaves are sprayed with 1 mL of *B. amyloquefaciens* suspension, then air-dried. The center of the leaf was dripped with 5 mL of 10^6 conidia/mL of *A. brassicicola* and then waited for 36 h. The leaf was then cut into around 5x5 mm and immersed twice in 1 mL alcohol 50% for 1 hour each. Leaf then washed with 600 µL 0.1 M phosphate buffer (1 M Na_2HPO_4 93.2 mL, 1 M NaH_2PO_4 6.8 mL in 1 L H_2O, pH 8.0) for 1 hour, then stained with 0.01% of aniline blue for 2 h. The callose deposition image analysis is assayed under the fluorescent microscope (Leica, Wetzlar, Germany). The fluorescents of the callose deposition are measured using Image J software, under a consistent threshold. Means of callose deposition were calculated from 5 samples/treatments in each of three independent experiments. The data were analyzed using Tukey's multiple range test ($p < 0.05$).

Results and Discussion

Inhibition Test

Our previous findings exhibit that application of two strains of *B. amyloliquefaciens* (PMB04 and PMB05) has strong antagonistic activity against plant pathogens and increases plants' immune system [25, 26]. In this study, we sought to evaluate whether these strains could be biocontrol agents against *A. brassicicola*. The dual culture technique of *B. amyloliquefaciens* strains PMB04 and PMB05 against *A. brassicicola* showed that both PMB04 and PMB05 bacterial cells were able to suppress the mycelium growth of *A. brassicicola* (Figure 1) on the agar plate. The inhibition rate of PMB04 was higher than PMB05, as PMB04 can decrease mycelium growth by up to 24.6%, while PMB05 can only suppress it by 22.69%. The inhibitory zone of PMB04 against *A. brassicicola* was slightly higher (7.38 mm) but not significantly different compared to PMB05 (6.72 mm). The inhibition rate of PMB04 and PMB05 was 30.0% and 15.6%, respectively, against *Colletotrichum gloeosporioides* [26]. This result is also proven by previous research, which reported that *in vitro* bioassay using the dual culture technique showed that three strains of *B. amyloliquefaciens* were able to suppress the mycelial growth of *F. verticillioides, R. stolonifera*, and *P. variabile* [14]. The reduced rate of pathogen growth was attributed to the disruption in the fungi's cell metabolism during hyphal expansion, which resulted in structural deformation [27]. Thus, the ability of antagonistic microorganisms to inhibit the mycelium growth of phytopathogens on agar media is directly proportional to the inhibition of spore germination of phytopathogens.

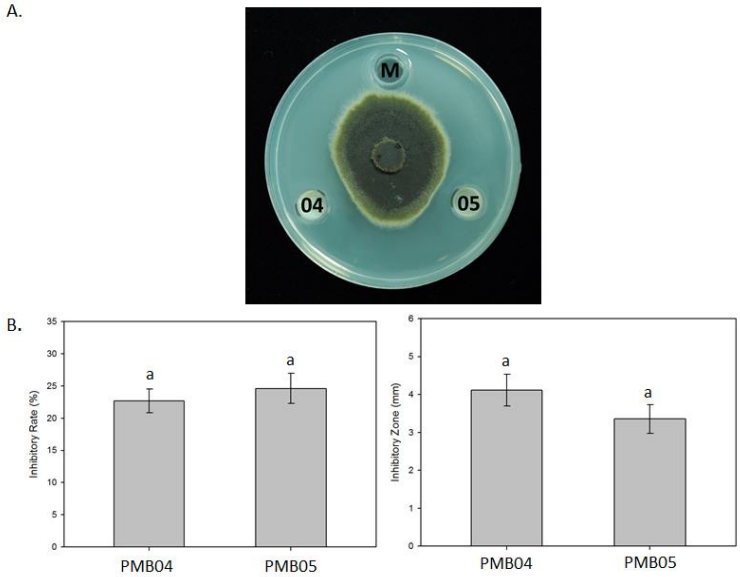

Figure 1. *In vitro* inhibitory effect of *Bacillus amyloliquefaciens* strains PMB04 and PMB05 against *Alternaria brassicicola* on PDA-NA media. (A) Phytopathogen *A. brassisicola* was grown for 36 hours, then 20 µL *B. amyloliquefaciens* strains were inoculated in the ring around the pathogen and incubated for 7 days at 28°C. M indicates MOCK treatment that is applied as the water control treatment. (B) Measurement of inhibitory rate and inhibitory zone of *B. amyloliquefaciens* against *A. brassisicola*. Values are the mean ± standard deviation for three repeats, and letters indicate the significant differences of each column based on Tukey's multiple range test ($p < 0.05$)

We found that the culture filtrate of PMB04 has a higher suppression (82%) of the spore germination of *A. brassicicola* compared to PMB05 (48%) (Figure 2). As illustrated in Figure 2A, treated spores with PMB04 and PMB05 were vacuolated and ruptured, preventing them from developing into normal mycelia. The other spores germinated abnormally, and the germination sites grew into circular bubbles. Recent research showed that *B. amyloliquefaciens* prevents over 50% of *Erysiphe cichoracearum* conidial germination [13]. In addition to suppressing spore germination, the presence of PMB04 and PMB05 creates swelling of non-germinating *A. brassicicola* conidia. Figure 2B shows that 18% and 52% of the swelling spores were found in the treatment with the cultural filtrate of *B. amyloliquefaciens* PMB04 and PMB05, respectively. Previously, subsequent application of *B. megaterium* on the abnormal germ tubes of *A. alternata* will also make them vacuolated and ruptured [28], also, *B. amyloliquefaciens* treatment resulted in wide breaches in the cell walls of *F. graminearum* conidia and hyphae, as well as serious damage to the plasma membranes [29].

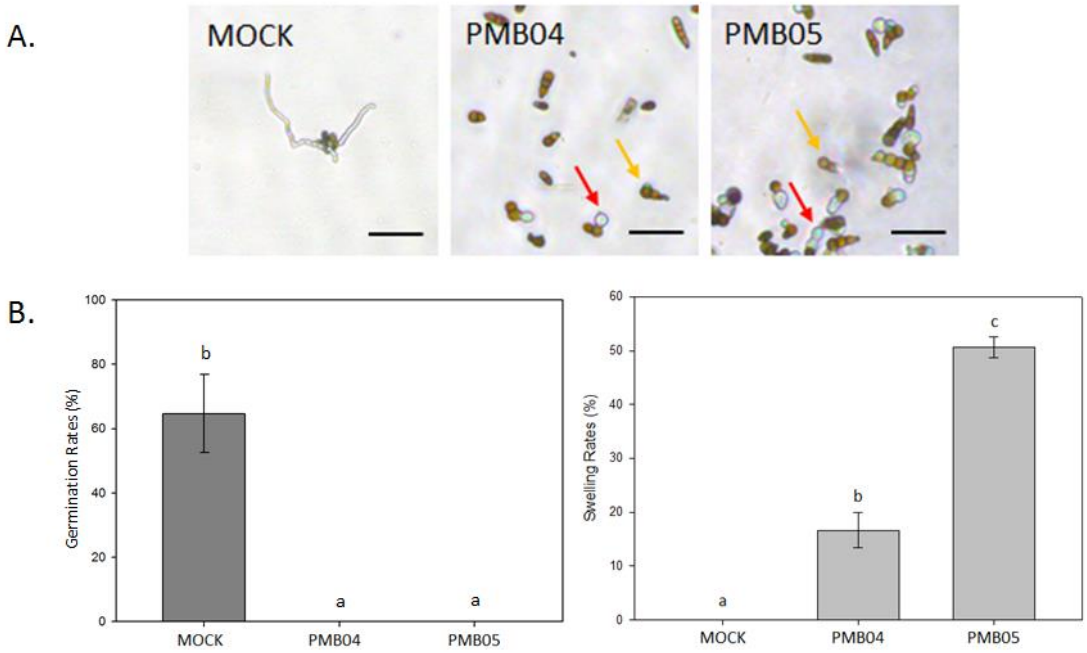

Figure 2. Effect of cultural filtrate from *Bacillus amyloliquefaciens* on conidial germination of *Alternaria brassicicola*. (A) The growth of *A. brasicicola* at 12 hours post-inoculated with *B. amyloliquefaciens* strains PMB04 and PMB05. The red arrow denotes spores that germinate abnormally by the formation of round bubbles in the germination sites, while the yellow arrow represents the swollen spores. The bar indicates 100 µm in length. (B) Conidial germination rates and swelling rates of *A. brassiscola* post-inoculated with *B. amyloliquefaciens* strains PMB04 and PMB05. Error bars indicate standard deviations of the mean for three repeats of Chinese cabbage infection. The letters above the bar indicate the significant differences based on Tukey's multiple range test ($p < 0.05$).

Those antagonistic activities of *B. amyloliquefaciens* are inextricably linked with its ability to create antifungal substances. Several scientific studies reported that different *B. amyloliquefaciens* strains co-produce lipopeptides, which are responsible for their antifungal action [13, 14, 30, 31]. Cyclic lipopeptides such as surfactins, fengycin, iturin A, and bacillomycin D have considerable antifungal properties of *B. amyloliquefaciens*. Iturins A is a well-known antifungal lipopeptide that is highly hemolytic and can inhibit fungi by causing holes in the pathogens' cell membranes, whereas fengycins are very effective against filamentous fungi such as *A. brassicicola*. In the other hand, bacillomycin D has high antifungal activity due to membrane permeability, and surfactins work together to enhance its antifungal activity [21]. In addition, iturin A caused the cellular contents of *F. graminearum* to leak and/or inactivate, causing deformation and conglobation along hyphae, and inhibiting the phytopathogen's branch development and proliferation [29], while fengycin induced vacuolation and conglobation in immature hyphae and branch tips of *F. graminearum*. Surfactin, a lipopeptide generated by this species, may enter phytopathogen cell membranes owing to its long-chain fatty acid, causing disintegration, damage, and breakdown of viral lipid membranes, resulting in antimicrobial action [31].

Biocontrol Efficacy

To understand the effects of *B. amyloliquefaciens* on controlling *A. brassiscicola*, two-week-old Chinese cabbage leaves were sprayed with *B. amyloliquefaciens* strains PMB04 and PMB05, then inoculated with *A. brassiscicola*. After 7 days, we found that black leaf spot symptoms were reduced due to the antagonistic effect of PMB04 and PMB05, compared to the control (Figure 3). The disease severity in the control treatment reached 53.0%, while PMB04 and PMB05 suppressed the disease severity up to 32.7% and 26.5%, respectively. This study suggested that *B. amyloliquefaciens* PMB04 and PMB05 are promising biological control agents for controlling black leaf spot disease. This is in line with the previous research which reported that severity of black rot disease in cabbage was highly reduced by *B. amyloliquefaciens* strain PMB04 (21.2%) and PMB05 (8.4%) [25]. Moreover, *B. amyloliquefaciens* was also reported to have high efficacy toward tobacco powdery mildew [13] and three fungal pathogens on maize [14].

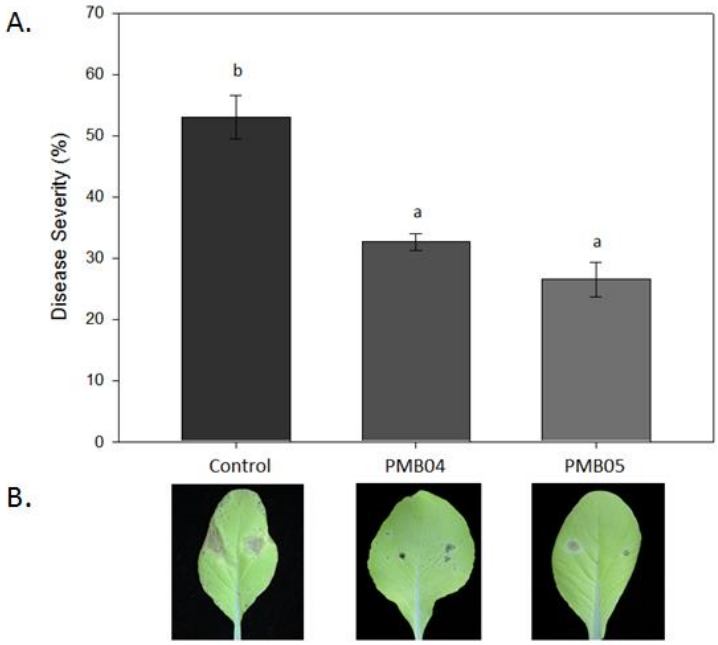

Figure 3. Control efficacies of *Bacillus amyloliquefaciens* against black leaf spot disease on Chinese cabbage leaves. (A) Disease severities were evaluated on leaves at 7 days post-inoculation (dpi). Error bars indicate standard deviations of the mean for three repeats of Chinese cabbage infection, and letters above the bars indicate the significant differences based on Tukey's multiple range test ($p < 0.05$). Stars indicated the significant difference between PMB04 and PMB05 treatments according to the t-test ($p<0.05$). (B) The symptom of black leaf spot disease on Chinese cabbage was reduced by *B. amyloliquefacienss* strains

In particular, we found that PMB05 outperformed PMB04 in terms of black leaf spot disease control. This defense can be offered by synthesizing antibiotics or competing for available resources and growth sites. *Bacillus* spp. interacts positively with plants by inhibiting phytopathogens through the synthesis of antimicrobial chemicals and competing for nutrients and space [21, 27, 32]. Beyond its activity as an antagonistic agent, *Bacillus* spp. induces plant immunological responses, increasing plant resistance to infection [21, 33, 34]. *Bacillus* spp. may also form biofilms, which improve their rhizosphere competence, promote soil fertility, and protect non-target species [33]. This combination of actions effectively protects plants while also boosting growth and general health.

Activation of Plant Immunity

To gain more insight into the induced immune system, the intensification of plant immune response was assayed by observing the callose deposition. Callose deposition is a cell wall reinforcement that is generated during plant-microbe interactions and can be easily triggered by the introduction of pathogen-associated molecular patterns and bacterial flagellin epitopes [22, 23]. During a pathogen invasion, callose is deposited between the cell membrane and the cell wall of plants. The thickening of the cell wall serves as a barrier to further prevent pathogen penetration, improving plant resistance against pathogens. The callose deposition could be detected by observing the blue fluorescent spot under fluorescent microscopy. Callose deposition that showed at treatment PMB04 and PMB05 appeared close to the spore.

In the experiment employing culture filtrate, the relative fluorescence intensity with PMB04 application was not significantly different from the blank treatment, while the relative fluorescence intensity with PMB05 treatment was 1,921.13. Furthermore, the bacterial cell of *B. amyloliquefaciens* shows a higher relative fluorescent intensity than the cultural filtrate. Bacterial cells of *B. amyloliquefaciens* PMB05 show callose deposition 12-fold higher than the cultural filtrate (Figure 4). These two experiments show that spraying bacterial cells on Chinese cabbage leaves may dramatically improve the plant's immune response, and PMB05 is the more effective of the two strains.

Callose deposition on the leaf of Chinese cabbage was only increased by the treatment of PMB05, followed by the inoculation of *A. brassisicola*. Meanwhile, healthy leaves sprayed with PMB04 and PMB05 did not exhibit a defense reaction (Figure 4). Given this condition, we may deduce that the plant's immune

response will be activated when the pathogen infects the guard cells on the leaves. On the contrary, PMB04 did not cause any intensified fluorescent signals in Chinese cabbage leaves. *B. amyloliquefaciens* strains PMB04 and PMB05 display two main biocontrol mechanisms, known as antagonism and induced systemic resistance. Here is the reason why, during the inhibitory assay performed with the dual culture assay and spore germination assay, we may see that the hyphal growth and spore germination were stopped in the presence of PMB04 and PMB05. Moreover, the callose deposition assay indicates that *B. amyloliquefaciens* PMB05 has an indirect inhibition towards the pathogen, compared to PMB04. A similar result was reported that PMB04 has a better *in vitro* inhibitory effect toward *Xanthomonas campestris* pv. *campestris*, but PMB05 has a better biocontrol efficacy and revealed that PMB05 activates plant basal defense through callose deposition [25]. The first common strategy of plant disease resistance is using pre-formed structures and substances such as plant cuticle surfaces, plant cell walls, antimicrobial chemicals, and peptides that inhibit or block pathogen-derived toxins/enzymes [22]. In addition, fungal infection causes plasmodesmata callose deposition, limiting the fungus's ability to propagate across cells [23]. Thus, callose deposition may be linked to an early plant defense mechanism against pathogen infection.

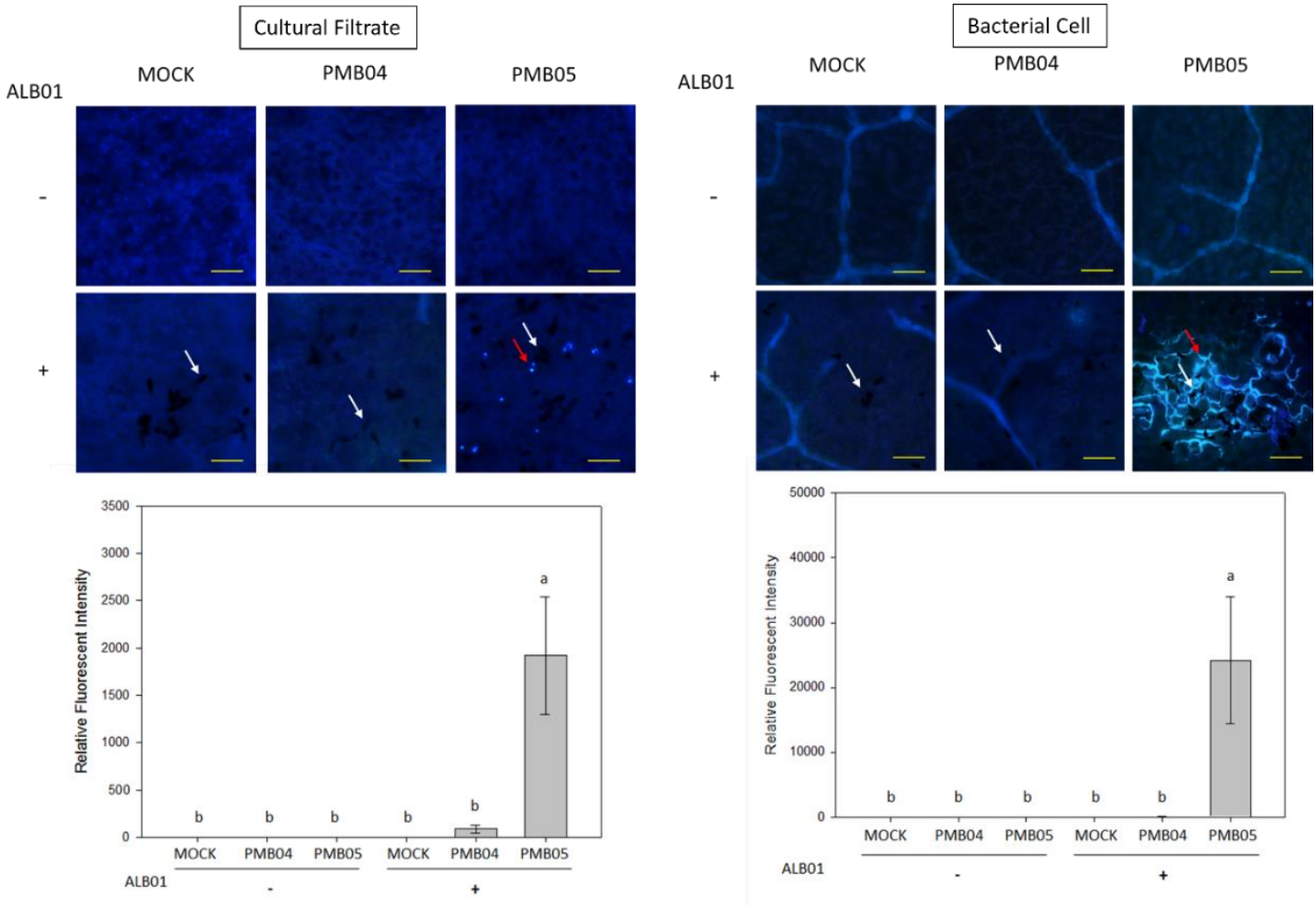

Figure 4. Callose deposition was intensified by the cultural filtrate and bacterial cell *Bacillus amyloliquefaciens* upon the inoculation of *Alternaria brassicicola* conidia on the leaf of Chinese cabbage. The sample is collected after 36 hours post-inoculation with *A. brassicicola*, stained with aniline blue, and observed under the fluorescence microscope. The white arrow shows conidia of *A. brassicicola*, and the red arrow shows callose deposition. The yellow bar indicates 100 µm in length. Relative fluorescent intensity calculated by ImageJ. The different letter above the bar indicates significant differences based on Tukey's multiple range test ($p < 0.05$)

Conclusions

The current findings reveal that *B. amyloliquefaciens* strains PMB04 and PMB05 could inhibit the mycelium growth and spore germination of *A. brassisicola*. *B. amyloliquefaciens* strains PMB04 and PMB05 were evaluated for successful biocontrol efficacy that significantly suppressed the infection of *A. brassisicola*. Interestingly, this research proved that *B. amyloliquefaciens* PMB05 can intensify the immune system with the appearance of callose deposition on the infected leaves.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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