J. Plant Med. Vol. 59 No. 3, 2017 39
DOI:10.6716/JPM.201709_59(3).0005

Application of *Bacillus amyloliquefaciens* to control black rot disease on cabbage caused by *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

Li'aini, A. S., Lin, Y.-H., Huang, T.-C., and Sulistyowati, L. 2017. Application of *Bacillus amyloliquefaciens* to control black rot disease on cabbage caused by *Xanthomonas campestris* pv. *campestris*. J. Plant Med. 59(3):39-44.

Black rot disease (BRD) caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is considered the most important disease of crucifers worldwide. Various strategies have been developed to control BRD on cabbage, including the use of antagonistic microorganisms. In this study, we tested the efficacy of antagonistic *Bacillus amyloliquefaciens* strains, PMB04 and PMB05, in controlling BRD of cabbage. We found these two antagonistic strains inhibited Xcc growth in dual culture assays and suppressed BRD by seed treatment. To gain more insights on the differential antagonism employing by PMB04 and PMB05, the callose deposition was assayed as an indication of the activation of plant defense response. The cabbage leaves treated with PMB05 showed increased callose deposition upon the inoculation of Xcc in comparison with the ones treated with PMB04 and water, suggesting the application of *B. amyloliquefaciens* PMB05 strongly suppressed the disease severity of BRD by activating plant basal defense and inhibiting Xcc growth, whereas *B. amyloliquefaciens* PMB04 greatly reduced the growth of Xcc in the seedlings. The results of this research indicate the development of *B. amyloliquefaciens* strains as biocontrol agents has a great potential to control BRD of cabbage in the future.

Keywords: *Bacillus amyloliquefaciens*, biological control, seed treatment, plant defense response, callose deposition

INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc) is the causal agent of black rot, a disease, accountable for the major loss of cruciferous crop worldwide [1, 2]. Substantial crop losses have been reported from the rapid spread of the bacteria under favorable conditions at all plant growth stages [2, 3]. Moreover, black rot disease (BRD) occurs in all parts of the temperate and subtropical zones of the world where rainfall or heavy dews are plentiful and average temperatures are between 25-30 ˚C [4]. This seed borne disease causes considerable yield losses up to 50% by premature defoliation and therefore causing severe economic losses [2, 3].

Various strategies have been developed previously to control BRD, including the use of Xcc-free seeds and transplants, hot water seed treatment, resistant varieties, plant extracts, cultural practices, physical, and chemicals [3, 5]. In addition, the use of antagonistic microorganisms is considered the most promising approaches for rational and safe plant protection due to their rapid growth, easy handling, aggressive colonization of rhizosphere, and environmental sustainability [1, 6].

Among antagonistic bacteria, *Bacillus* strains have the ability to form endospores that can easily be formulated and stored; even more, these spores are tolerant against adverse environments [7]. A strain of *Bacillus subtilis* has been known to reduce BRD symptom on cabbage and cauliflower both in dry and rainy season in Zimbabwe [8]. Moreover, *B. amyloliquefaciens* strains have been reported to significantly suppressed anthracnose on mulberry leaves, fungal wilt on tomato, bacterial wilt on tomato and tobacco and stem rot on cucumber [9-13]. Our preliminary studies exhibit that two *B. amyloliquefaciens* strains, PMB04 and PMB05, were able to control bacterial fruit blotch on watermelon and anthracnose on strawberry. Especially, these diseases were significantly reduced by *B. amyloliquefaciens* strain PMB05 through induced resistance associated with strong callose deposition. In this study, we assayed

the two *B. amyloliquefaciens* strains PMB04 and PMB05 for controlling BRD on cabbage. By inhibitory assay, monitoring bacterial population in seedlings, and plant defense response, the mechanisms of these two antagonistic strains on black rot disease of cabbage were evaluated.

MATERIALS AND METHODS

Bacterial strains and cultural conditions

Bacillus amyloliquefaciens strains PMB04 and PMB05 were used as the antagonistic bacteria, while *Xanthomonas campestris* pv. *campestris* (Xcc) strain Xcc62 was used as the pathogen in this study. All of the bacterial strains were provided by Laboratory of Bacteriology, Department of Plant Medicine, National Pingtung University of Science and Technology, Taiwan. All bacterial strains were cultured on nutrient agar plate (NA, 10 g of glucose, 5 g of peptone, 5 g of yeast, 3 g of beef extract 15 g of agar, at pH 7.0 in 1 L) at 28 ˚C for 2 days as microbial sources.

Inhibitory assay of *Bacillus amyloliquefaciens* against *Xanthomonas campestris* pv. *campestris*

To assess the inhibitory effect of *B. amyloliquefaciens* strains against the BRD pathogen, the dual culture assay was performed on NA. Bacterial suspension of antagonistic strains and pathogen was prepared with sterile distilled H_2O and adjusted to OD_{600} at 0.3 (equivalent to 10^8 CFU/ml) by spectrophotometer (CromTech, CT-2800, Taiwan). Firstly, the bacterial suspension of Xcc was sprayed on NA. Then, two sterile filter papers were put on the surface of NA and further dripped 10 μl of each bacterial suspension of PMB strains on the filter papers. After incubation at 26˚C for 3 days, the inhibitory zones were measured and calculated using a caliper as follow: Inhibition zone (mm) = (the average diameter of clear zone – diameter of antagonistic strain)/2. This experiment was performed with 15 replicates in each treatment. The data were analyzed by *t*-test at 5% significance level.

Seed treatments and inoculation

This experiment was conducted to assay the ability of *B. amyloliquefaciens* strains PMB04 and PMB05 to suppress the disease of Xcc-contaminated seeds of cabbage. Bacterial suspensions of Xcc or antagonistic strains were prepared to OD_{600} at 0.3 in the sterile distilled water containing 0.5 % (w/v) of carboxymethyl cellulose (CMC). Firstly, the seeds soaked in Xcc suspension for 24 h, the air-dried seeds were regarded as contaminated seeds. Then, the contaminated seeds were soaked in bacterial suspension of each *B. amyloliquefaciens* for 24 h. The treated seeds were further planted in the greenhouse to evaluate the biocontrol efficacy by disease incidence (DI) and disease severity (DS). DI and DS were counted from 10 plants as one repeat, and five repeats were carried out in one treatment. The evaluation was conducted at 10, 20, and 30 days after planting. DI and DS were calculated as described below: DI (%) = (the number of diseased plants/ total number of plants) 100%; DS (%)= [∑(the number of diseased plants in an index × disease index)/ the number of observed plants × the highest disease index] × 100%. Disease index that used in this experiment consists of six severity scale: 0, no symptom; 1, water soaked symptom; 2, yellowing symptom; 3, the characteristic symptom; 4, necrosis on the whole leaf; and 5, wilt symptom [8]. The data were analyzed by the analysis of variance with Tukey HSD test at 5% significance level.

Population assay of *Xanthomonas campestris* pv. *campestris* from plants

To assay the population of Xcc established on the seedlings affected by antagonistic strains, the *B. amyloliquefaciens* strain-treated contaminated seeds were used. Three seedlings from each treatment were taken to assay the population of Xcc from leaves and stems. Each part of the seedlings was sampled (0.1 g) at 20- and 30-day post planting. The ground tissues were put into 900 μl of sterile ddH_2O to obtain the crude extract. A 100 μl crude extract was taken to evaluate the population of Xcc. Data were analyzed by the analysis of variance and analyzed with Tukey HSD test at 5% significance level.

Callose deposition

The bacterial suspensions of Xcc62, PMB04 and PMB05 were prepared as described above. To assay the callose deposition, the leaves of 30-day old plant were used. First, the bacterial suspension of each antagonistic strain at OD_{600} of 0.3 was infiltrated in the leaves. After 30 minutes, the suspension of Xcc62 was infiltrated at the same area. After 8 hours, leaves were sampled and stained with alanine blue to observe the callose deposition in the leaves. Staining of callose was performed according to the method described by Su *et al* [14]. Briefly, the sampled leaves was cut into tiny pieces and then immersed in 95% alcohol to discolor the leaves. After 24 h, leaves were washed by 5 ml of 0.1 M phosphate buffer (pH) for 3 times. The leaves were stained with 0.01 % of aniline blue (Sigma, USA) in 0.1 M phosphate buffer in the dark for 2 h. The leaves then observed under the fluorescence microscope with an excitation at 340 – 380 nm and emission at 400 – 425 nm filter set (Leica, Germany). The relative fluorescent intensity was calculated by the software ImageJ (https://imagej.nih.gov/ij/). The values were normalized by the blank treatment inoculated with Xcc62 alone. The data were analyzed by

Tukey HSD test at 5% significance level.

RESULTS

In vitro inhibitory assay of *Bacillus amyloliquefaciens* against *Xanthomonas campestris* pv. *campestris*

The inhibitory effect of *B. amyloliquefaciens* strains against *X. campestris* pv. *campestris* (Xcc) strains was assessed to demonstrate that the growth of Xcc could be inhibited by *B. amyloliquefaciens*. The result showed that both antagonistic strains were able to inhibit the growth of Xcc62 on nutrient agar plate (Fig. 1A). The average inhibitory zone done by PMB04 and PMB05 were 6.23 mm and 4.06 mm, respectively. In addition, PMB04 exhibited stronger inhibitory effect than PMB05 (Fig. 1B).

Biocontrol efficacy of *B. amyloliquefaciens* on control black rot disease on cabbage

In this study, seed treatment was carried out to assay the biocontrol efficacy of *B. amyloliquefaciens* on BRD of cabbage. As a result, disease incidence (DI) and disease severity (DS) of BRD were significantly suppressed by PMB04 and PMB05 than those in control treatment. DI on cabbage was reduced to 19.0 % and 4.0 % by the application of PMB04 and PMB05, respectively, compared to 89.0 % of the control treatment (Fig. 2A). Moreover, PMB05 showed a better suppression on the occurrence of BRD than PMB04. The DS was reduced to 21.2 % and 8.4 % by the application of PMB04 and PMB05, respectively, compared to 58.4 % of the control treatment (Fig. 2B). Similarly, seed treatment with PMB05 almost completely inhibited the occurrence of symptom (Fig. 2C). In addition, PMB05 exhibited stronger suppression on BRD than PMB04.

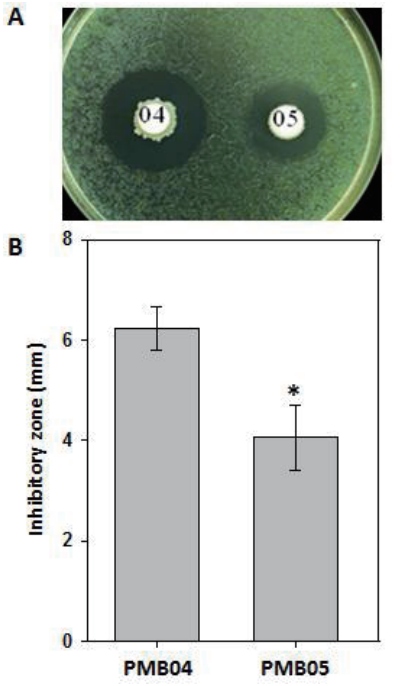

Fig. 1. Inhibitory effect of *Bacillus amylolyquefaciens* against *Xanthomonas campestris* pv. *campestris* on nutrient agar plate. A, Confrontation assay showed the inhibitory zone around the filter disks that were inoculated with *B. amylolyquefaciens* PMB04 and PMB05. B, The measurement of the inhibitory zone shown in A. The asterisk above the bar indicates significant difference in the inhibitory effect of *B. amylolyquefaciens* strains PMB04 and PMB05 against *X. campestris* pv. *campestris* strain based on the *t*-test ($p < 0.05$).

Population of *X. campestris* pv. *campestris* in the seedling of

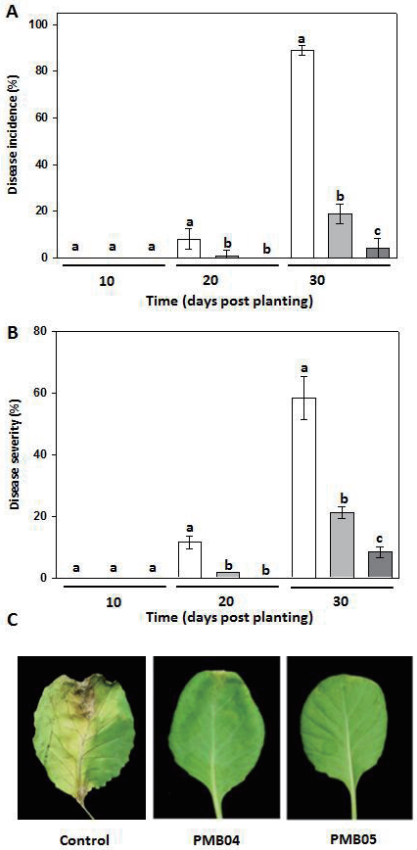

Fig. 2. Black rot disease on cabbage reduced by seed treatment of *Bacillus amyloliquefaciens* strains. Panel A and Panel B reveals the disease incidence and disease severity, respectively, at 10-, 20-, and 30- day post planting in the peat moss. White bars indicate seeds are inoculated with *X. campestris* pv. *campestris* only. Light grey bars indicate seeds are treated with *B. amyloliquefaciens* strain PMB04 after the inoculation of *X. campestris* pv. *campestris*. Dark grey bars indicate seeds are treated with *B. amyloliquefaciens* strain PMB05 after the inoculation of *X. campestris* pv. *campestris*. Different letters above the bars indicate significant difference among the treatments at 10, 20 and 30 days after planting according to the Tukey HSD test ($p < 0.05$). Panel C reveals the symptom of black rot disease on cabbage reduced by *B. amyloliquefacienss* strains at 30 days post planting.

cabbage

The population of Xcc in the leaves and stems of cabbage was enumerated at 20- and 30- days post planting. As shown in Figure 3, the population of Xcc in the leaf and stem of the blank treatment was significantly higher than those in the treatments with PMB04 or PMB05. Moreover, the population of Xcc in seedlings treated with PMB04 was significantly lower than that with PMB05. At 30-days post planting, the population of Xcc in the leaves of cabbage in control plants, plants treated with PMB04, and plants treated with PMB05 were 36.33, 1.70, and 9.85×10^5 CFU/g fresh tissue, respectively (Fig. 3A). Meanwhile, the population of Xcc in the stems of cabbage in control plants, plants treated with PMB04, and plants treated with PMB05 were 29.94, 1.59, and 5.81×10^5 CFU/g fresh tissue, respectively (Fig. 3B).

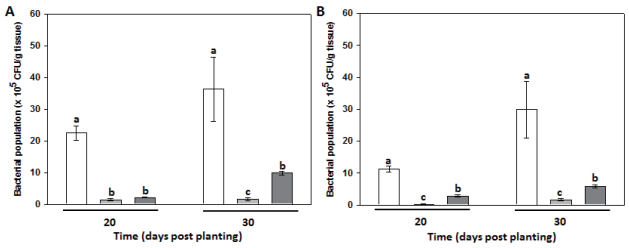

Fig. 3. Population of *Xanthomonas campestris* pv. *campestris* in the seedling of cabbage. Seeds are coated with B. amylolyquefaciens strains after the inoculation of *X. campestris* pv. *campestris* strain Xcc62. Bacterial populations in the leaves (A) and stem (B) of cabbage are determined at 20 and 30 days post planting. White bars indicate seeds are inoculated with *X. campestris* pv. *campestris* only. Light grey bars indicate seeds are treated with B. amyloliquefaciens strain PMB04 after the inoculation of *X. campestris* pv. *campestris* strain Xcc62. Dark grey bars indicate seeds are treated with *B. amyloliquefaciens* strain PMB05 after the inoculation of *X. campestris* pv. *campestris* strain Xcc62. Different letters above the bars indicate significant difference among the treatments at 20 and 30 days after planting according to the Tukey HSD test ($p < 0.05$).

Callose deposition on cabbage leaf

The callose deposition could be detected by observing the blue fluorescent spot under fluorescent microscopy. Results exhibited that the callose deposition on the leaf of cabbage was greatly increased by the treatment of PMB05 followed by Xcc62 inoculation (Fig. 4). The fluorescent intensity was strongly induced to 2.61-fold in the treatment with PMB05 when compared to the blank treatment with strain Xcc62 alone. On the contrary, PMB04 did not cause any intensified fluorescent signals and intensity (0.93-fold) in cabbage leaves.

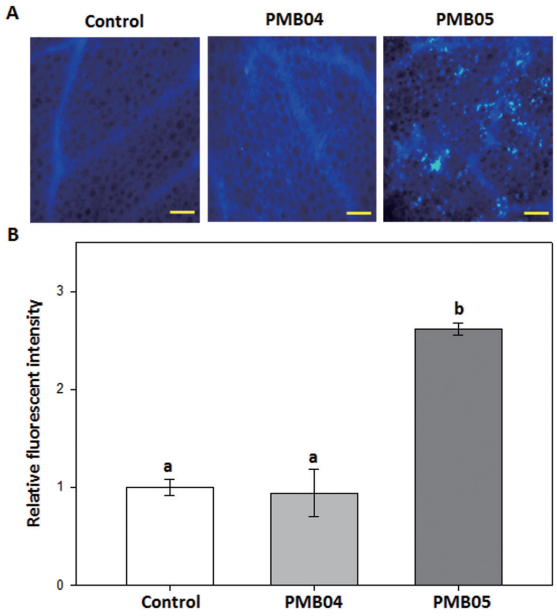

Fig. 4. Callose deposition induced by *Bacillus amyloliquefaciens* strain PMB05 upon inoculation of *Xanthomonas campestris* pv. *campestris* strain Xcc62 on the leaves of cabbage. The inoculation of *X. campestris* pv. *campestris* is performed at 30 minutes after the infiltration of individual *B. amyloliquefaciens* strain PMB04 or PMB05. The leaves are collected at 8 hours after treatment. Panel A exhibits the images of leaves stained with 0.01% aniline blue to observe callose deposition. The scale bar indicates 20 μm in length. Panel B reveals the relative fluorescent intensity calculated by ImageJ. Different letter above the bar indicates significantly different according to Tukey HSD test ($p < 0.05$).

DISCUSSION

In recent years, biological control of plant pathogen has received increasing attention as a promising method for rational and safe plant protection. Most of the studies used *in vitro* screening to isolate antagonistic microorganism that is able to produce toxic metabolites against pathogens for further control of plant diseases were promising [9, 11, 15]. In this study, *in vitro* inhibitory assay showed that the growth of Xcc62 was inhibited by *Bacillus amyloliquefaciens* strains PMB04 and PMB05 (Fig. 1). Therefore, we suggested that these two *B. amyloliquefaciens* strains could be used to control black rot disease (BRD) of cabbage.

Bacillus strains are effectively antagonistic against a broad spectrum of plant pathogens and they can be used in various ways or in the integrated management [16]. Additionally, the application of antagonistic microorganisms on seed provides an ideal delivery system to reduce plant pathogens in the rhizosphere where they are active [17, 18]. The occurrence of BRD on cabbage is often due to the

infested seed [8, 19]. In this study, seed treatment was performed to assay for the efficacy of *B. amyloliquefaciens* on controlling BRD of cabbage. Results showed that the application of *B. amyloliquefaciens* on Xcc-contaminated seeds strongly suppressed BRD on cabbage. We suggested that the use of *B. amyloliquefaciens* by seed coating method could be used as a promising strategy to control BRD. This protection can be mediated by the production of antibiotics or competition for available nutrients and growth spaces [9, 20]. In this study, the populations of Xcc in plant parts treated with PMB04 were significantly lower than those treated with PMB05. This result was positively correlated the result of *in vitro* inhibitory assay. However, the disease incidence and disease severity carried out in the treatment with PMB05 showed a better suppression against Xcc62 on cabbage than PMB04. Therefore, we suggested that *B. amyloliquefaciens* PMB05 may employ other mechanism in addition to antagonism to reduce BRD on cabbage. Previously, our preliminary results found that the induction of plant resistance against bacterial fruit blotch on watermelon could be achieved by applying PMB05. In addition, this induced resistance is associated with the callose deposition. Callose deposition is regarded as an effective barrier at the site of pathogen attack during the relatively early stages of PAMP-triggered immunity [21, 22]. Here, we demonstrated that callose deposition on cabbage was strongly intensified by the cells of PMB05 upon the inoculation of Xcc62. In contrast, there was no increase of the deposited callose in the treatment with PMB04 or blank treatment. Thus, we suggested that plant immune response elicited by Xcc could be intensified by PMB05.

In this study, the results supported that BRD could be controlled by *B. amyloliquefaciens* strains by the antagonistic effect. Most importantly, *B. amyloliquefaciens* strain PMB05 exhibited dual activities in controlling cabbage black rot disease by the inhibition of Xcc growth and the intensification of plant defense, indicating that PMB05 is a good candidate as a biocontrol agent for integrated pest management.

ACKNOWLEDGEMENTS

This work was supported by grants to Dr. Yi-Hsien Lin from the Ministry of Science and Technology, Taiwan (MOST- 104-2311-B-020-001 and MOST 105-2628-B-020 -002-MY3).

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摘　要

李安妮、林宜賢、黃卓治、Sulistyowati, L.。2017。應用*Bacillus amyloliquefaciens*防治由*Xanthomonas campestris* pv. *campestris*所引起之甘藍細菌性黑腐病。植物醫學 59(3)：39-44。

甘藍細菌性黑腐病是由 *Xanthomonas campestris* pv. *campestris* (Xcc) 所引起，且被認為在全世界是十字花科中最重要的病害之一。目前許多研究已證明可利用拮抗微生物來防治此病害的發生。本研究之目的為利用土壤分離之 *Bacillus amyloliquefaciens* PMB04 及 PMB05 菌株來評估其是否具有防治甘藍黑腐病能力。首先在平板拮抗活性分析中，PMB04 及 PMB05 菌株皆對於 Xcc 不同菌株均有抑制的效果。進一步將預先接種有Xcc之種子分別處理上述兩種拮抗菌菌株來評估其對甘藍黑腐病之防治效果。結果顯示，此兩種拮抗菌株對甘藍黑腐病的罹病率與罹病度均有顯著的抑制效果，其中又以PMB05菌株的處理為佳。然而在種子處理後，PMB04菌株相較於PMB05菌株可大幅降低幼苗中病原菌族群。為了進一步了解此些拮抗菌株在誘導抗病上的活化，利用癒傷葡聚醣的觀察進行分析。結果顯示僅PMB05菌株能夠在同時接種有Xcc的情況下加強此植物防禦反應之訊號，推測PMB05菌株對黑腐病之抑制效果可能與其在植物防禦訊號的提升有關。綜上所述，本研究說明在防治甘藍黑腐病上可利用具有拮抗效果或提升植物防禦反應的 *B. amyloliquefaciens* 菌株為可行的策略。

關鍵詞：液化澱粉芽孢桿菌、生物防治、抑制活性、種子處理、植物防禦反應。