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Potential of *Bacillus* spp. Isolated from Food Waste Compost for Controlling Rice Diseases

(Potensi *Bacillus* spp. yang Dipencilkan daripada Kompos Sisa Makanan untuk Mengawal Penyakit Beras)

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ABSTRACT

Bacterial leaf blight, bakanae and blast are severe, economically damaging rice diseases caused by *Xanthomonas oryzae*, *Fusarium fujikuroi,* and *Magnaporthe oryzae*, respectively. *Bacillus* spp. have been applied as bioactive, eco-friendly agents to control these diseases. In this study, five antagonistic strains isolated from food waste compost, namely *B. subtilis* strain BS, *B. amyloliquefacien* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus.* sp strain 3-BA were tested for their efficacy against rice diseases. The inhibition of *X. oryzae* was tested by paper disc diffusion, while the inhibitions of *F. fujikuroi* and *M. oryzae* were tested in dual cultures. It was found that *B. amyloliquefacien* strain C2-1 gave the widest clear zones. At 24 h, 48 h, 72 h and 7 days, the strain had produced zones of inhibition against *X. oryzae* of 7.41±0.65, 7.9±0.20, 8.8±0.65 and 8.90±0.12 mm, respectively. *B. amyloliquefacien* strain C2-1 also reduced the growth of the fungal rice pathogens *F. fujikuroi* and *M. oryzae*, achieving 98.79% and 97.74% inhibitions, respectively. *B. amyloliquefaciens* strain C2-1 was also effective against *X. oryzae, F. fujikuroi,* and *M. oryzae* in the greenhouse. *Fourteen days after spraying rice plants with the B. amyloliquefacien* strain C2-1*, infections with X. oryzae, F. fujikuroi, and M. oryzae were inhibited by* 60*%,* 37*%,* and 25*%, respectively.* The results suggested that *B. amyloliquefacien* strain C2-1 can be used as a biocontrol agent against bacterial leaf blight, bakanae, and rice blast diseases.

Keywords: *Bacillus amyloliquefacien*; food waste; *Fusarium fujikuroi; Magnaporthe oryzae; Xanthomonas oryzae*

ABSTRAK

Hawar daun bakteria, bakanae dan letupan adalah penyakit padi yang teruk dan merosakkan ekonomi yang disebabkan masing-masing oleh *Xanthomonas oryzae, Fusarium fujikuroi* dan *Magnaporthe oryzae*. *Bacillus* spp. telah digunakan sebagai agen bioaktif, mesra alam untuk mengawal penyakit ini. Dalam kajian ini, lima strain antagonis diasingkan daripada kompos sisa makanan iaitu strain BS *B. subtilis*, strain C2-1 *B. amyloliquefacien*, strain 1-BA *B. tequilensis*, strain 2-BA *B. licheniformis* dan strain 3-BA *Lysinibacillus* sp. telah diuji keberkesanannya terhadap penyakit padi. Perencatan *X. oryzae* telah diuji dengan resapan cakera kertas, manakala perencatan *F. fujikuroi* dan *M. oryzae* diuji dalam dua kultur. Didapati bahawa strain C2-1 *B. amyloliquefacien* memberikan zon jelas terluas. Pada 24 jam, 48 jam, 72 jam dan 7 hari, strain tersebut telah menghasilkan zon perencatan terhadap *X. oryzae* masing-masing 7.41±0.65, 7.9±0.20, 8.8±0.65 dan 8.90±0.12 mm. Strain C2-1 *B. amyloliquefacien* juga mengurangkan pertumbuhan patogen beras kulat *F. fujikuroi* dan *M. oryzae,* masing-masing mencapai 98.79% dan 97.74% perencatan. Strain C2-1 *B. amyloliquefaciens* juga berkesan terhadap *X. oryzae, F. fujikuroi* dan *M. oryzae* dalam rumah hijau. Empat belas hari selepas pokok padi disembur dengan strain C2-1 *B. amyloliquefacien*, jangkitan dengan *X. oryzae, F. fujikuroi* dan *M. oryzae* masing-masing direncat sebanyak 60%, 37% dan 25%. Keputusan menunjukkan bahawa strain C2-1 *B. amyloliquefacien* boleh digunakan sebagai agen biokawalan terhadap penyakit hawar daun bakteria, bakanae dan letupan padi.

Kata kunci: *Bacillus amyloliquefacien; Fusarium fujikuroi; Magnaporthe oryzae;* sisa makanan; *Xanthomonas oryzae*

Introduction

Rice (*Oryza sativa* L.) is a staple food for half of the world's population. Majority of the rice produced globally is grown in Asia (Bin Rahman & Zhang 2023). Thailand is one of the three largest exporters of rice, supplying various countries around the world (Yusiana et al. 2022). Among the major problems facing rice producers are diseases of rice. The main rice diseases in Thailand, which cause severe yield losses, are bacterial leaf blight disease, bakanae disease and rice blast disease.

Bacterial leaf blight disease is caused by the bacterial pathogen *Xanthomonas oryzae*. A pathogen variation study suggested that breeding with a single gene might not be effective and biological control is therefore the better option for eco-friendly management of the disease (Naqvi 2019). Bakanae disease is a seed-borne disease caused by the fungus, *Fusarium fujikuroi*. Infected rice flowers will produce infected seeds that transfer the disease to the next plant generation. The common symptoms are leaf abnormalities (Saito et al. 2021). Rice blast disease is caused by the fungus, *Magnaporthe oryzae*. Losses due to this disease are estimated at around 6% of the global rice harvest every year (Eseola et al. 2021). *Magnaporthe oryzae* also damages other economically important crops, such as maize and several herbaceous plants (Muthayya et al. 2014; Thakur et al. 2009).

Modern approaches to the management of crop diseases have led to the application of eco-friendly bioproducts and the development of biological control agents as safe alternatives to chemicals. *Bacillus* spp. are particularly interesting in this regard. They secrete secondary metabolites with anti-fungal and antibacterial properties and siderophores, which contribute to the effectiveness of these strains (Khan et al. 2022). *Bacillus* spp. have been tested as biocontrol agents against various phytopathogens. For example, *B. amyloliquefaciens* was an effective antagonistic bacterial strain that could control bacterial leaf blight disease and fungal plant diseases (Cho & Kasem 2018; Ji et al. 2013). *B. subtilis* applied as a rice seed treatment was able to control the narrow brown leaf spot blast (He et al. 2019), and *Lysinibacillus* sp. has been tested as a biological control of sheath blight (Shabanamol et al. 2021). In addition, Araya et al. (2021) found that four isolates of *Bacillus* spp. reduced the plant disease caused by *Bipolaris* spp. by 75.79–81.81%. Massawe et al. (2018) reported that volatile organic compounds (VOCs) produced by *Bacillus* spp. inhibited the mycelial growth of *Sclerotinia sclerotiorum*, and VOCs emitted by the *B. subtilis* CL2 strain inhibited the hyphal growth of pathogenic fungi. Dhitikiattipong et al. (2011) reported that powders of *Bacillus* spp. reduced bakanae disease and increased rice yield. Yenjit, Pengphol and Intana (2020) reported that spraying *B. subtilis* NS–03 successfully controlled narrow brown spot in Patumthanee 1 rice in the greenhouse.

Recently, we isolated five *Bacillus* spp. from food waste compost. The species included *B. subtilis* strain BS, *B. amyloliquefacien* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus* sp. strain 3-BA. In order to develop bioproducts for biological control of rice diseases, we studied the efficacy of these isolates in controlling the rice pathogens *X. oryzae*, *F. fujikuroi,* and *M. oryzae.* The most effective *Bacillus* sp. was further tested on infected rice plants in the greenhouse.

Materials and Methods

Biocontrol agents and pathogens

Bacillus subtilis strain BS, *B. amyloliquefaciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus* sp. strain 3-BA, isolated from food waste compost, were obtained from the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The rice pathogens *X. oryzae*, *F. fujikuroi,* and *M. oryzae* were obtained from Phattalung Rice Research Center, Phattalung, Thailand.

Inhibition assays for antagonistic activities of *Bacillus* spp. against *Xanthomonas oryzae*

The five antagonistic *Bacillus* spp. and the rice pathogens were cultivated in nutrient glucose broth medium (NGB; 13 g of nutrient broth and 12.5 g of glucose anhydrous per 1 L of distilled water). A 100 mL working volume of the NGB medium was used in each 250 mL Erlenmeyer flask for cultivation. The optical density of cell suspensions was measured at 600 nm (OD_{600}) with a UV-vis spectrophotometer (Thermo Fisher Scientific, USA). Initial cell suspension density was equal to 0.2 at OD_{600}. The culture flasks were inoculated with 10% (v/v) inocula and incubated at 25 °C for 24 h on a rotary shaker set at 130 rpm, until cell suspension densities reached 0.5 at OD_{600}. The testing of bacterial antagonism was done using

the agar diffusion method on agar plates (Balouiri, Sadiki & Ibnsouda 2016). A basal layer was prepared by pouring 10 mL of glucose agar (NGA) medium into a Petri dish that was then placed under a laminar flow hood to cool. Meanwhile, an intracellular suspension of pathogenic bacteria, or seed layer, was prepared by adding 1 mL of *X. oryzae* to 10 mL NGA in tubes. The tubes were heated to approximately 45 to 50 °C, then agitated in a vortex mixer. The seed layer was then poured over the basal layer and allowed to harden for 2-3 hours. These plates were used for testing the bacterial inhibition of pathogens by the paper disc diffusion method (Lehtopolku et al. 2012; Pengnoo et al. 2000). In this method, the agar plate was inoculated with a standardized inoculum of each antagonistic *Bacillus* sp. by dropping 10 µL of the *Bacillus* sp. on four antibiotic test paper discs (about 6 mm in diameter). The discs were placed in separate quadrants of the seed layer. The control was 10 µL of sterile water dropped on an antibiotic test paper disc that was placed in the center of the seed layer. The Petri dishes were then incubated at 37 °C for 24 h, and the diameters of growth inhibition zones were measured. The zones were quantified using Equation (1). The *Bacillus* sp. that gave the largest inhibition radii was classified as the most effective *Bacillus* sp. and was used in further studies at inhibition times of 48 h, 72 h, and 7 days.

$$Ra = (Dc - Ds) / 2 \quad (1)$$

where Ra is the inhibition radius (mm); Dc is the diameter of the clear area (mm); and Ds is the diameter of the specimen (mm).

ANTAGONISTIC ACTIVITIES OF *Bacillus* spp. IN FUNGAL INHIBITION ASSAYS

The fungal pathogens were cultivated on potato dextrose agar (PDA) (Himedia, India) plates and incubated for 7 days at 25 °C. Five antagonistic *Bacillus* spp. were tested for their inhibition of two fungal pathogens. The *Bacillus* spp. were prepared as they were in the previous section and cultivated in NGB until the cell suspension density was 0.5 at OD_{600}. Each *Bacillus* sp. was streaked at the edges of a Petri dish as thin lines about 3 cm in length, while a mycelial plug (diameter 5 mm) from an actively growing edge of a fungal colony was deposited at the center of the plate. The distance between both inocula was at least 2.5 cm. The inoculated plates were then incubated at 25 °C for 7 days (Khabbaz & Abbasi 2014). Inhibition of the two fungal pathogens was reported as the percent inhibition of radial growth, calculated using the following formula (Boonrayong et al. 2023) for percentage reduction of mycelial colony expansion, and compared to control plates that were not inoculated with *Bacillus* spp.

$$\text{Percent inhibition} = (R_1 - R_2) / R_1 \times 100 \quad (2)$$

where R_1 is the radial growth of fungus on the control plate; and R_2 is the radial growth of fungus towards the antagonist.

PATHOGEN TESTING UNDER GREENHOUSE CONDITIONS

The most effective *Bacillus* sp. was applied to inhibit rice diseases in Sangyod rice cultivated in greenhouses. The Sangyod rice seeds were planted in 434 wells filled with peat moss. When the rice seedlings were 20 days old, they were transplanted into 10-inch planting pots, placed in the experimental greenhouse at the Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand, and grown from October 2021 to February 2022. The most effective *Bacillus* sp. was tested for its efficacy against the studied rice diseases in a completely randomized design (CRD) study comprising three treatments, each with five replicates, for a total of 15 pots as follows: Treatment 1: Control (sprayed with distilled water for disinfection), Treatment 2: Sprayed with the effective *Bacillus* spp. only, and Treatment 3: Inoculated with a pathogen and sprayed with the effective *Bacillus* spp.

Rice leaves were exposed to *X. oryzae* by the clipping method (Kauffman 1973). *Xanthomonas oryzae* was cultured in NGB medium until the initial cell suspension density was equal to 0.5 in its OD_{600}. Inoculation scissors were sterilized in 70% EtOH before use. *Xanthomonas oryzae* was inoculated by cutting rice leaves about 3 cm from the tips, creating wounds that allowed the pathogen to enter the rice plant from the scissors. About five leaves per plant were inoculated. Pathogenicity was assessed for 21 days after inoculation before the effective *Bacillus* sp. was sprayed on the plants (10^{12} CFU/mL, 100 mL/pot). Meanwhile, the pathogenic fungi were tested in accordance with a method for fugal inoculation and evaluation of rice diseases (Park et al. 2008). Rice plants harvested at the late tillering stage were inoculated with *F. fujikuroi,* and *M. oryzae* by placing a mycelial ball beneath the leaf

sheath. The inoculated sheath was covered immediately with aluminum foil. When lesions appeared after three days, the aluminum foil was removed and the infected rice plants were covered with clear plastic to enhance humidity for 21 days, helping the disease develop. Lesion lengths on the sheath of inoculated plants were measured for 7 days after inoculation. After 21 days, the infected plants were sprayed with the effective *Bacillus* spp. strain (10^{12} CFU/mL, 100 mL/pot). The percentage of relative lesion height (% RLH) of both pathogenic bacteria and pathogenic fungi were calculated using the following formula according to Kumar et al. (2009):

$$\text{Percentage of relative lesion height (RLH)} = (\text{lesion height}/\text{plant height}) \times 100 \quad (3)$$

STATISTICAL ANALYSIS

The results are shown as mean values with standard deviations. The homogeneity of variance was assessed, then the analysis of variance (ANOVA) combined with the Tukey honestly significant differences (HSD) test was used to analyze parametric data, and the Kruskal-Wallis test combined with the *post hoc* Dunn test was used to analyze non-parametric data. The significance level was set at 5% ($p < 0.05$) for all tests. The statistical analyses were conducted using the IBM SPSS Statistics program version 26.0.

RESULTS AND DISCUSSION

CHARACTERIZATION OF POTENTIAL ANTAGONISTIC BACTERIA

In order to characterize potential antagonistic bacteria, *B. subtilis* strain BS, *B. amyloliquefaciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus* sp. strain 3-BA were tested against *X. oryzae*. At 24 h, the inhibition assay showed that only *B. amyloliquefaciens* had produced clear zones. However, the clear zones were not as distinct as desired. Consequently, inhibition was studied at different time points, using the OD_{600} of *B. amyloliquefaciens* at 0.5 instead of 0.2.

The inhibitory activity of *B. amyloliquefaciens* strain C2-1 was studied at 24 h, 48 h, 72 h, and 7 days. The observed clear zones measured 7.41±0.65, 7.90±0.20, 8.80±0.65, and 8.90±0.12 mm, respectively (Figure 1). This result indicated that *B.*

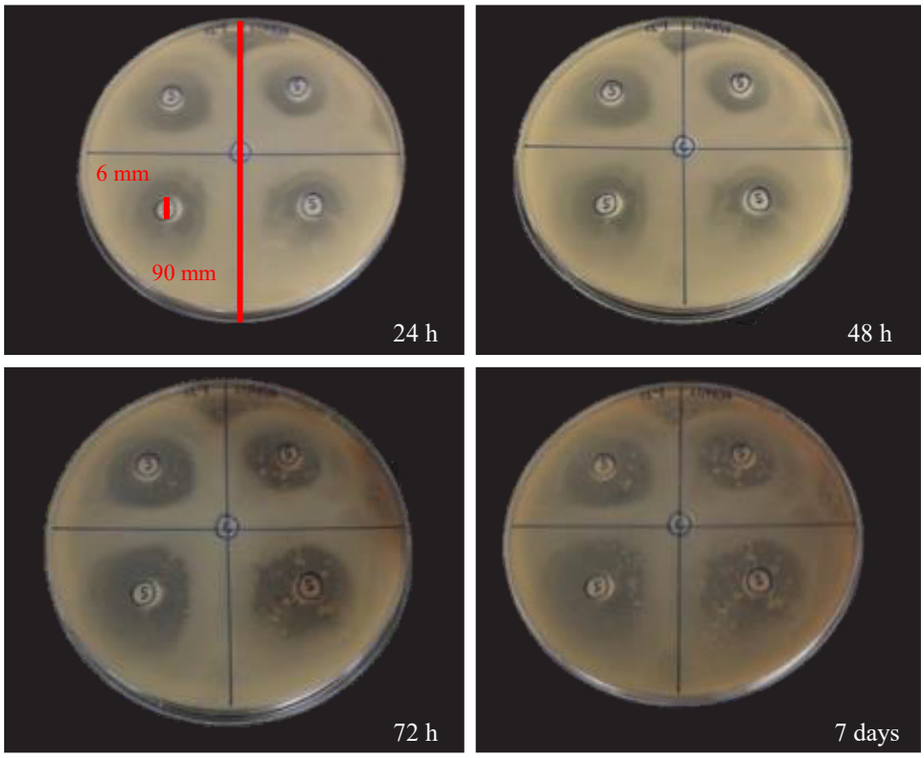

FIGURE 1. *Xanthomonas oryzae* was inhibited by *B. amyloliquefaciens* strain C2-1 applied at OD 0.5 (OD_{600}) in a disc diffusion assay. The plates were incubated at 37 °C. Inhibition zones were measured as shown at 24, 48, and 72 h, and 7 days after inoculation

amyloliquefaciens strain C2-1 could exert antibacterial activity against *X. oryzae* for at least one week. Wu et al. (2015) reported that *B. amyloliquefaciens* FZB42 had antibacterial activity against *X. oryzae* rice pathogens and Cho and Kasem (2018) reported that *B. amyloliquefaciens* S20A1 was an effective control against bacterial leaf blight disease of rice in Thailand. Therefore, *B. amyloliquefaciens* strain C2-1 could potentially be used as an alternative control agent against bacterial leaf blight disease.

ANTIFUNGAL ACTIVITY OF ANTAGONISTIC *Bacillus* spp. STRAINS

The antagonistic *Bacillus* spp. showed antifungal activity against both rice pathogenic fungi in dual culture tests incubated for 7 days. As seen in Figure 2, *B. amyloliquefaciens* strain C2-1 showed stronger inhibitory activity toward mycelial growth than the other *Bacillus* spp. *B. amyloliquefaciens* strain C2-1 inhibited *F. fujikuroi* by 98.79%, followed by *B. licheniformis* strain 2-BA (53.65%). *B. amyloliquefaciens* strain C2-1 inhibited *M. oryzae* by 97.74%, followed by *Lysinibacillus* sp. strain 3-BA (59.7%). The results show that *B. amyloliquefaciens* strain C2-1 suppressed the fungal pathogens more effectively than the other *Bacillus* spp. and was therefore studied in further antifungal tests for 9 and 14 days. After incubation for 7, 9, and 14 days, the growth regions of the pathogenic fungi were reduced in size and were surrounded by *B. amyloliquefaciens* strain C2-1 (Figure 3). Furthermore, the pathogenic fungi were cultured with *B. amyloliquefaciens* strain C2-1 and observed under a conventional light microscope (at 100X) (Figure 4). Mycelia were distorted, and constricted, while the mycelia in the controls were normally elongated. The results were consistent with the findings of Ji et al. (2013), who tested *B. amyloliquefaciens* CNU114001 against fungal plant diseases. Under the microscope, they observed abnormal, swollen and curved mycelia at the boundary. Sungtong, Pengnoo and Boonyapipat (2021) also reported that the deformation of mycelia of pathogenic fungi was probably caused by the strong adhesion of *Bacillus* spp. to the mycelia. Bacterial adhesion caused the fibers to atrophy, exhibiting surface wrinkling and clearly abnormal morphology.

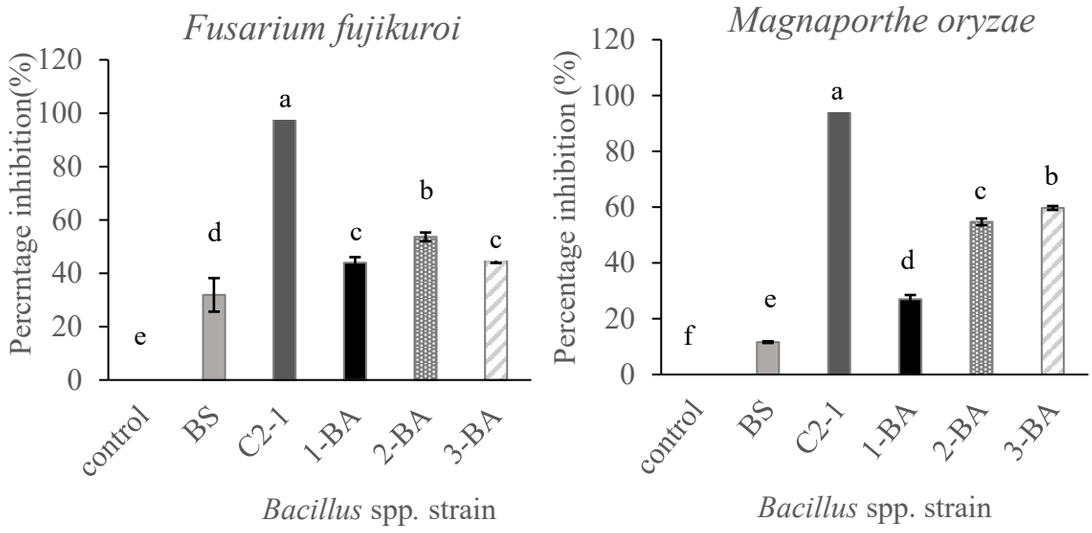

FIGURE 2. Percentage inhibition of mycelial growth of *Fusarium fujikuroi* and *Magnaporthe oryzae* by *Bacillus subtilis* strain BS, *B. amyloliquefaciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus.* sp strain 3-BA. Inhibition was determined on dual culture plates incubated for 7 days at room temperature

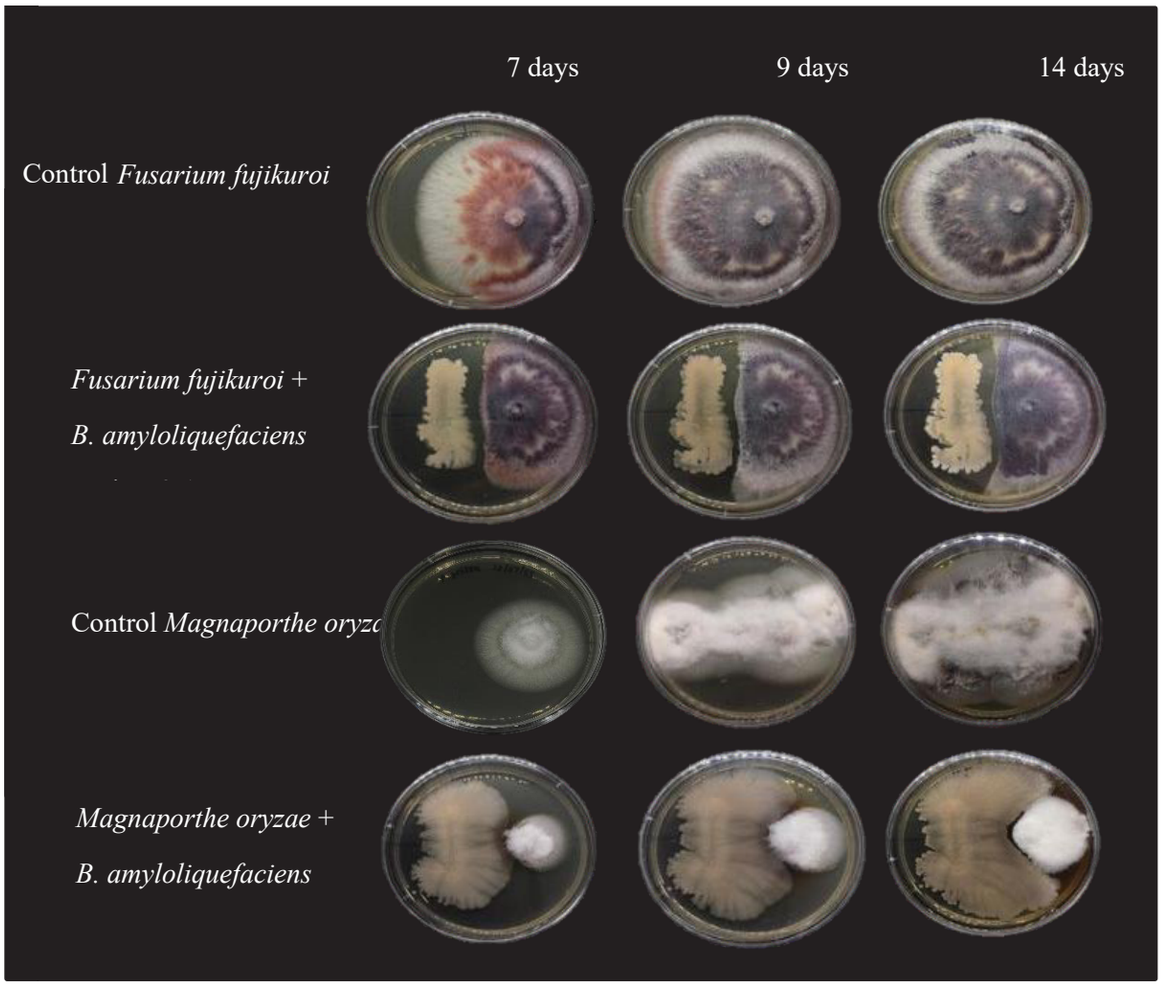

FIGURE 3. Effects of *Bacillus amyloliquefaciens* strain C2-1 against *Fusarium fujikuroi* and *Magnaporthe oryzae* on dual culture plates incubated for 7, 9, and 14 days at room temperature

TESTING EFFICIENCY OF PATHOGEN INHIBITION UNDER GREENHOUSE CONDITIONS

The effects of *B. amyloliquefaciens* strain C2-1 against rice pathogens under greenhouse conditions are shown in Table 1. The results show that *14 days after spraying infected rice plants with B. amyloliquefaciens* strain C2-1, the severity of infections with *X. oryzae, F. fujikuroi* and *M. oryzae* were reduced *by* 60%, 37%, *and* 25%, *respectively*. The lesions from infection were reduced in size. This result demonstrates that *B. amyloliquefaciens* strain C2-1 reduced infestation with the bacterial pathogens and inhibited the spread of the disease to neighboring plants (Kanjanamaneesathian et al. 2007).

CONCLUSION

In conclusion, *B. amyloliquefaciens* strain C2-1 isolated from food waste compost showed good potential to inhibit the bacterial growth of *X. oryzae*, and the mycelial growth of *F. fujikuroi,* and *M. oryzae* in both the laboratory and greenhouse. In the laboratory, *B. amyloliquefacien* strain C2-1 produced clear inhibition zones of 7.41±0.65, 7.9±0.20, 8.8±0.65 and 8.90±0.12 mm against *X. oryzae* at 24 h, 48 h, 72 h, and 7 days, respectively. *Fusarium fujikuroi* and *M. oryzae* were inhibited by 98.79% and 97.74%, respectively, in the presence of *B. amyloliquefacien* strain C2-1. In the green house, 14 days after treatment by spraying, *B. amyloliquefaciens* strain C2-1 had reduced the severity of infections with *X. oryzae*, *F. fujikuroi,* and *M. oryzae* by 60%, 37%, and 25%. Thus, *B. amyloliquefaciens* strain C2-1 can be used as an alternative strain to control bacterial leaf blight, bakanae and rice blast diseases.

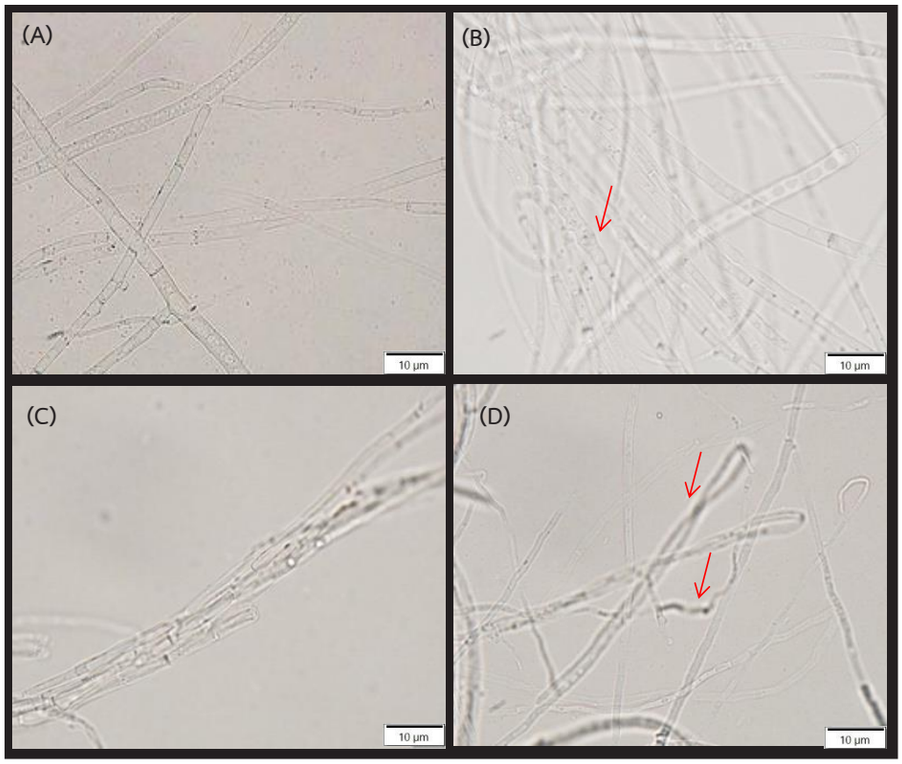

FIGURE 4. Morphological characteristics of *Fusarium fujikuroi* and *Magnaporthe oryzae* mycelia on dual culture plates at 7 days; (A) control *F. fujikuroi*; (B) *F. fujikuroi* + *B. amyloliquefaciens* strain C2-1; (C) control *M. oryzae*; (D) *M. oryzae* + *B. amyloliquefaciens* strain C2-1. Scale bars indicate 10 µm on images from a compound microscope (100X). The arrows point to swollen balloon-like abnormalities

TABLE 1. Characteristics of *B. amyloliquefaciens* strain C2-1 against *X. oryzae, F. fujikuroi* and *M. oryzae* in the rice greenhouse before and after treatment

Condition	Bacterial leaf blight disease	Bakanae disease	Rice blast disease
	Xanthomonas oryzae	*Fusarium fujikuroi*	*Magnaporthe oryzae*
Before			
After			
Severity (% Relative lesion height, RLH)	60	37	25

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