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A Strong Antifungal-producing Bacteria from Bamboo Powder for Biocontrol of *Sclerotium rolfsii* in Melon (*Cucumis melo* var. *amanta*)

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Abstract

Bamboo (*Bambusa edulis* Munro) rhizosphere has been known for its potency to reduce plant pathogen because it contains diverse beneficial microbes, thus bamboo plant itself might be a potential source for bio-control agent discovery. Sclerotium rot, caused by *Sclerotium rolfsii* Sacc, is one of the most devastating non-specific diseases of plant, including melon (*Cucumis melo* var. *amanta*). The aim of this study was to screen and characterise novel bacteria isolates from bamboo powder that have beneficial application for crop protection. *Bacillus subtilis* BMB26 produced an extracellular metabolite that effective against a wide range of pathogenic fungi, including *S. rolfsii*, and resistant to heat and protease. Furthermore, to evaluate the application of cell-free filtrates BMB26 on melon plants, a cotyledon test was done under greenhouse condition. A significant decrease was observed in disease incidence (up to 77%) occurred in BMB26-treated melon leaves inoculated with *S. rolfsii* after 4 days post-inoculation. This result showed that *Bacillus subtilis* BMB26 has a potential application as a bio-control agent against phytopathogenic fungi.

Keywords: *Bacillus subtilis*; *Sclerotium rolfsii*; Southern blight rot; Antifungal compound; Antagonistic assay

Introduction

Fungal diseases cause extensive yield loss in important crops thus becoming one of major challenges facing agriculture. *Sclerotium rolfsii* Sacc, causal agent of Sclerotium rot (SR), is one of destructive plant pathogens distributed worldwide [1]. This pathogen is known to infect more than 400 different plant species, including melon (*Cucumis melo* var. *amanta*), and as high as 30-70% of SR incidence has been reported in melon in Korea [2]. Controlling of this fungal pathogen has always been very difficult due to its survival structure (sclerotia) that remains viable in soil for several years and limited control of fungicide application [3]. Therefore, it is necessary to find alternative methods of SR disease control. In this problem, the use of antagonistic microorganisms would be a cost-effective way to control this fungal pathogen. Furthermore, antifungal compound from bio-control microorganisms have certain advantages over conventional chemical fungicides. They are environmental friendly and non-toxic to human health.

Bamboo (*Bambusa edulis* Munro) is a multifunctional plant and one of the most important plants in Asia. In Indonesia, bamboo rhizosphere has been known as a disease suppressive soil and has been used as seedling growth medium by local farmers [4]. This phenomenon may be caused by its diverse bacteria community, which can reflect the soil microbial abundance [5]. It is known that plant species plays an important role in influencing the diversity of soil microbial community due to the release of organic compound [6], thus there are also possibilities that the bamboo plant itself is an untapped resource for discovery of antifungal-producing bacteria. In recent years, plant microbes have become important in the study of novel antimicrobial compound that has a high potential use in agriculture [7,8].

Although several bacteria species are known for their antifungal activity, *Bacillus* spp. is one of the promising antifungal-producing bacteria because it can form endospore also can tolerate extreme pH, temperature, and osmotic condition. Therefore, from an ecological perspective, their application is sustainable (long term) [9]. Moreover, *Bacillus* spp. regarded as safe biological agents and their potential is considered high, due to diverse range modes of actions including antibiosis, production of siderophores, cell wall degrading enzymes, and lipopeptides producer [10]. *Bacillus subtilis*, one of most well-studied *Bacillus* species, is known to have an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds [11]. Among these antimicrobial compounds, cyclic lipopeptides of the surfactin, iturin, and fengycin families have been well recognized for their function and potential uses in biotechnology application [12].

In this present study, efforts have been made to isolate the antifungal-producing *Bacillus* spp. from bamboo powder in order to find bioactive compounds to control *S. rolfsii*. Both *in vitro* and *in vivo* bioassays were conducted to investigate the characteristic of bioactive compounds produced by these isolates and evaluate the possibility of those compounds to supress SR disease in melon. In addition, preliminary identification of the antimicrobial peptide compound has been done using HPLC and molecular study.

Material and Methods

Identification and isolation of antifungal-producing bacteria

An antifungal-producing bacterium used throughout this study, named BMB26, was isolated from bamboo powder, which was produced by crushing the bamboo into a fine powder form in a wood crusher (AMS-MFS600, China). Five grams of bamboo powder was homogenized into 45 ml of 2% saline solution. Following homogenization, samples were incubated in 80°C for 15 minutes to kill non-spore forming bacteria. Each sample was serially diluted until the concentration achieved 10^{-4} dilution and 100 µl of the 10^{-2}

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to 10^{-4} dilution were spread on Potato Dextrose Agar and King's B agar then incubated at 30°C ± 2°C overnight. *Bacillus*-liked colonies were collected and subcultured to new agar medium. These isolates were identified by the sequencing and phylogenetic analysis of 16S ribosomal RNA (16S rRNA) genes. 16S rRNA gene amplification was carried out with universal primers (Table 1) and 16S rRNA sequences were compared with GenBank database using Geneious software v6.0 for searching the closest matching sequence.

In vitro antifungal study on pathogenic fungal growth

The *in vitro* antagonistic assay against several phytopathogenic fungi was performed using dual culture test [9] with some modifications. The fungal cultures used in this experiment, *Sclerotium rolfsii, Ganoderma boninense*, *Pythium sp., Colletotrichum sp., Fusarium sp.,* and *Curvularia sp*., were obtained from Wilmar Benih Indonesia Research and Development Laboratory (Cikarang, Indonesia). All fungal cultures were maintained on Potato dextrose agar (PDA) at 30°C ± 2°C. For the assay, BMB26 isolate was streaked into PDA plate 2 cm from the 2 edges of the petri dish, and a 1×1 cm^2 of fresh mycelial plug was placed in the centre of the petri dish. Antagonistic test was also performed with agar well diffusion assay with some modification [13], using cell-free supernatant against the same phytopathogenic fungi. Isolates were grown in liquid medium at 30°C 4 days. The broth was centrifugated 8000 rpm for 2 minutes. The supernatant were collected and heated at 95°C for 10 min. A 6-mm diameter well was made in both sides of the agar plate, then 100 µl of boiled supernatant were subsequently poured into one of the well, while 100 µl of the growth medium was poured in the other well as a control. The plates were then incubated in the room temperature for several days. The zone of inhibition was measured for the antifungal activity.

Dose efficacy of culture filtrates against *S. rolfsii* growth *in vitro*

The inhibitory effects of these filtrates towards *S. rolfsii* were assessed using modified agar pour method by Ren et al. [14]. Antifungal product from medium production was centrifuged 8000 rpm for 5 minutes to separate the cell, then filtered with 0.22 µm milipore. Various concentrations of filtrates (1%, 2%, 5%, 10%, and 20%) were then mixed with a sterile, molten PDA and poured on sterile petri dish. Next, mycelial plugs (1×1 cm^2) from a 7-days-old fungal culture were placed in the center of each plate. The fungal growth was observed every day and the diameter of the fungal growth was measured in the end of experiment.

In vivo antifungal study against *s. rolfsii* in melon

Biocontrol efficacy of BMB26 was tested in green house study using cotyledon test, which modified from Purnamasari et al. [15]. Melon seeds were grown in 30 cell-trays (85 ml Kwikpot Tray™, each cell 55 mm diameter) containing soil mixture. Four seeds per genotype were sown in each cell and thinned to two seedlings per cell after emergence. Seedlings were grown until cotyledons were equivalent to growth stage 1.00 00 on the scale of Sylvester-Bradley and Makepeace [16], which is around ten days in melon. The trays were then placed in 35-liter clear plastic storage boxes so that high humidity could be maintained after inoculation as outlined below.

Inoculum of *S. rolfsii* was prepared by scrapping the 3-day-old mycelium of this pathogen off the surface of 9-cm Petri dish cultures, suspended in 50 mL Potato dextrose broth (PDB) containing 0.002% Tween® 20, and blended using a hand-held blender for 3 min. The macerated mycelial concentration was adjusted with the same liquid medium to 10^5 fragments ml^{-1} using a haemocytometer (SUPERIOR®, Berlin, Germany). A 10 µL droplet of BMB26 free-cell supernatant was deposited on each cotyledon of melon plants using a micropipette. After it dried, the same amount of *S. rolfsii* mycelial suspension was spotted in the same spot as the crude extract. Deionized water, *E. coli* free-cell filtrate, and PPC were deposited on the control plants. The extract of *E. coli* was used as negative bacteria control, while PPC, which is an antifungal compound from *Bulkhorderia sp*., was used as positive bacteria control. The mycelial suspension and bacteria supernatant were shaken regularly to maintain a homogenous mixture when inoculating the plants. After the inoculation, plants were incubated in the wet container to maintain high humidity and placed in green house. The infection was observed 96 h post inoculation (hpi) using 0-5 scale modified from Purnamasari et al. [15], where 0 = no visible symptoms, 1 = necrotic hypersensitive, 2 = necrotic and/or water-soaked lesion in inoculated spot, 3 = necrotic and/or water-soaked lesion outside

Gene	Name of primer	Sequence	Annealing temp.	size	Reference
Iturin D	ituD_F	ATGAACAATCTTGCCTTTTTA	50	1203	[18]
	ituD_R	TTATTTTAAAATCCGCAATT			
BacilomycinC	BacC_F	GAAGGACACGGCAGAGAGTC	60	875	[19]
	BacC_R	CGCTGATGACTGTTCATGCT			
FengycinD	FenD_F	GGCCCGTTCTCTAAATCCAT	60.1	269	[20]
	FenD_R	GTCATGCTGACGAGAGCAAA			
SurfactinA	SrfA_F	TCGGGACAGGAAGACATCAT	60.4	201	[20]
	SrfA_R	CCACTCAAACGGATAATCCTGA			
BacylisinA	BacA_F	CAGCTCATGGGAATGCTTTT	60.1	498	[20]
	BacA_R	CTCGGTCCTGAAGGGACAAG			
SubtilinS	SpaS_F	GGTTTGTTGGATGGAGCTGT	59.6	375	[21]
	SpaS_R	GCAAGGAGTCAGAGCAAGGT			
16S	16S_63F	CAGGCCTAACACATGCAAGTC		1324	[22]
	16S_926R	CCGTCAATTCCATTTRAGTTT			

Table 1: List of primers that used in this experiment.

inoculated spot, 4 = necrotic and/or water-soaked lesion (> 50%), and 5 = collapsing of cotyledon tissue with masses of mycelium. This 0-5 disease scores were converted into a Disease Severity Index (DSI) using the equation of Mohd Zainudin and Abdullah [17], where:

$$\text{DSI}\ (\%) = \frac{\Sigma\ (\text{A} \times \text{B}) \times 100}{\Sigma\ (\text{B}) \times 5}$$

Where A is the disease scores (0,1,2,....5, respectively) and B is the number of plants showing that disease scores per treatment.

HPLC analysis of BMB26 antifungal compound

For initial characterization, the active compound of BMB26 was checked for its stability in high temperature and high concentration of protease. The free-cell supernatant was heated 100°C for 15, 30, and 45 minutes or treated with 10 mg, 100 mg and 500 mg proteinase-K. For protease treatment, the mixture was incubated in 37°C for 1 hour, and then heated at 90°C for 5 minutes to inactive the enzyme. Then, the treated supernatant was tested using yeast for rapid antifungal test. Furthermore, the bioactive compound was further extracted by method reported by Grover et al. [18]. The filtered sterilized supernatant was added with 6 N HCl until the medium reached pH 2. After that, the broth was stirred in 4°C for 60 min to precipitate the antifungal compound and recovered by centrifugation (13000 rpm for 2 minutes at 4°C). The pellet was extracted with methanol. This methanol extract was dried under vacuum condition and used for HPLC studies. A reverse phase HPLC technique was used for detection of the active compound [19]. Sample analysis was performed using Agilent HPLC instrument (series 1200) with a binary pump, 717 plus auto-injector, 996 photodiode array detectors and 470 fluorescence detectors. The stationary phase consisted of ZORBAX 300SB C-18 column (150 × 4.6 mm internal diameter with 5 µm packing; Agilent). For mobile phase, acetonitrile: water (70:30) at 1 ml/min flow rate was used. HPLC analysis was performed at wavelength of 220 nm, which was detected for absorption maxima using photodiode array. 50 µl of sample was injected into HPLC under standardized conditions. Each run was repeated twice. The active compounds were collected and tested using yeast [20-22].

Amplification of antimicrobial peptide genes

Genomic DNA of *Bacillus* strain was extracted using Wizard® Genomic DNA Purification Kit (Promega) and electrophoretic analysis in agarose gel was performed according to standard protocols. Primers used for amplification of lipopeptide genes involved are shown in Table 1. For each sample, 50 µl reaction mixture was prepared in dH_2O using 25 µL GoTaq® Green Master mix (Promega) and10 µM primers (1 µl of each). Amplification reaction was accomplished with C1000™ thermal cycler (Bio-Rad) with the cycle conditions described in Table 1.

Statistical Analysis

A completely randomized design with four replications was used for the greenhouse experiment and the entire experiment was repeated once. The %DSI for cotyledon test was analysed using *one-way ANOVA with the statistical program R version 3.2.1. Tukey's honestly significant different test* ($P < 0.05$) was used to determine which means among the treatments differ from the rest.

Results

Identification and isolation of antifungal-producing bacteria

86 *Bacillus* isolates were isolated from the bamboo powder. After antifungal activity assay, only one isolate, BMB26, showed the highest inhibition of *S. rolfsii* mycelial growth and was used for further study. Morphologically, this isolate appeared Gram positive, rod-shaped, motile and single cells under light microscope. Taxonomically, the strain BMB26 was related to *B. subtilis* (100% similarity), *B. amyloliquefaciens* (100% similarity), and *B. methylotrophicus* (100% similarity) respectively on the basis of the 16S rRNA gene comparison.

In vitro antifungal study on pathogenic fungal growth

To gain a better understanding of the effectiveness of BMB26's antifungal compound against diverse phytopathogens, live bacteria cells and cell-free supernatant were tested against a variety of well-known pathogens (Figure 1). For all of the tested pathogens, mycelial growth inhibition and inhibition zones formation were observed (Figure 1A). Growth inhibition was variable for each fungus, with the strongest antagonistic activities was obtained by live BMB26 cells against *Ganoderma sp*. (97 ± 0.5%). Furthermore, both live cells and cell-free supernatant inhibited these phytopathogens to a similar degree; however, the cell-free supernatant completely lost its ability to inhibit *Pythium sp*. and *Collectotricum sp.* (Figure 1B). In regard to the melon pathogen *S. rolfsii*, it was noticeable that the mycelial growth was affected by this strain, although a less prominent inhibition zone was formed (Figure 1).

In addition, the dose effectivity of BMB26 supernatant extract was also tested against *S. rolfsii*. After 4 days of incubation, the mycelial growth of this pathogen was inhibited when the supernatant of BMB26 was added to the fungi cultivation medium (Figure 2). When supplemented with 10% of BMB26 supernatant extract, the mycelia diameter was approximately 14 times smaller than the control. Furthermore, *S. rolfsii* plugs were taken from plates where this pathogen had been exposed to BMB26 and were sub-cultured onto fresh PDA agar in order to examine the characteristic of this antifungal compound. The result showed that the sub-cultured *S. rolfsii* grew normally without the presence of the bacteria or its supernatant (data not shown) (Figure 2).

In vivo plant inoculation study

The green house experiment was performed to compare the abilities of BMB26 crude extracts, *Escherichia coli* extract (negative bacteria control), and PPC (*Bulkhorderia sp*. antifungal compound-positive bacteria control), to reduce the symptoms and development of SR disease in cotyledon. Typical of SR symptom, small necrotic and/or soft watery lesions, was detected in untreated melon after 2 days post inoculation (dpi). After 4 dpi, high disease incidence (100%) was observed in the non-treated plants; however, only 23% of disease incidence caused by *S. rolfsii* infection appeared in BMB26 treatment (Figure 3). The disease incidence of BMB26 showed equal effectiveness in controlling disease as PPC, while percent incidence in melon with *E. coli* treatment was similar to the untreated plants (Figure 3).

Characterization of BMB26 antifungal compound

To further examine the characteristic of BMB26 antifungal compound, the stability of cell-free supernatant against various concentrations of proteinase-K and high temperature were tested. The activity of this compound remained stable when proteinase-K was added until 500 µg/ml and after heated 100°C for 15, 30, and 45 min. This compound was then extracted using HCl precipitation and methanol evaporation. Upon RP-HPLC, six peaks were obtained from the BMB26 crude extract, with four of the peaks inhibited the growth of yeast (Figure 4). These four peaks had retention times of 21.65 min, 22.39 min, 23.02 min, and 23.85 min respectively. In addition, several

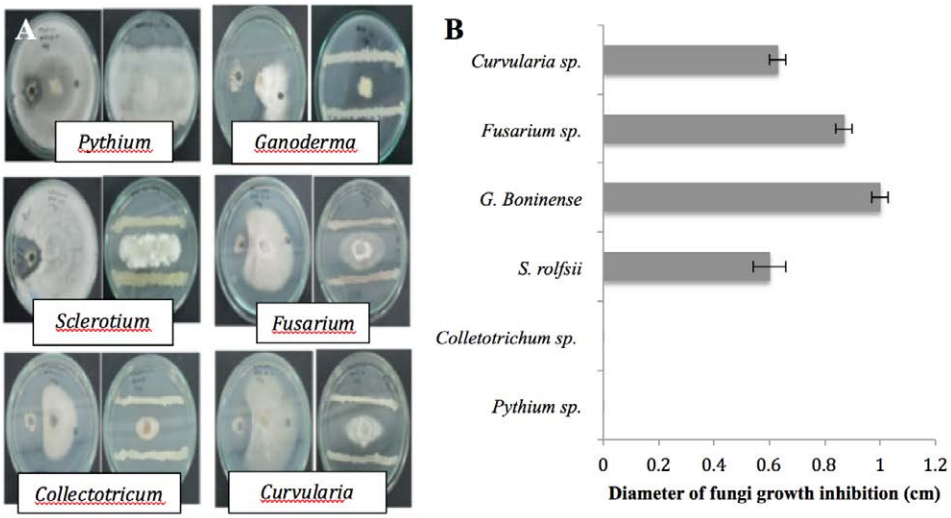

Figure 1: Inhibitory effect of BMB26 against several pathogenic fungal (A) Live cells of BMB26 (B) BMB26 cell-free supernatant.

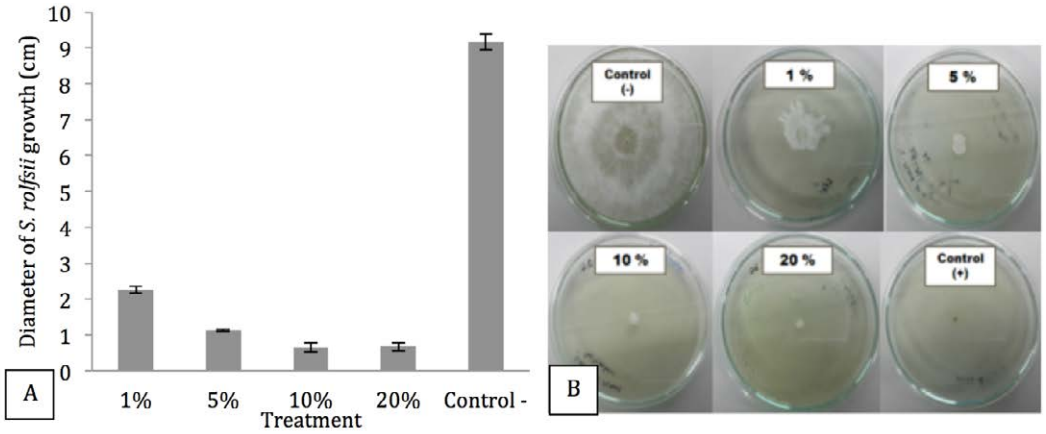

Figure 2: Inhibition of *Sclerotium rolfsii* by BMB26 with pour agar method (A) the growth of *S.rolfsii* on PDA with BMB26 supernatant after 4 days of incubation (B) Appearance of Sclerotium hyphae on this medium.

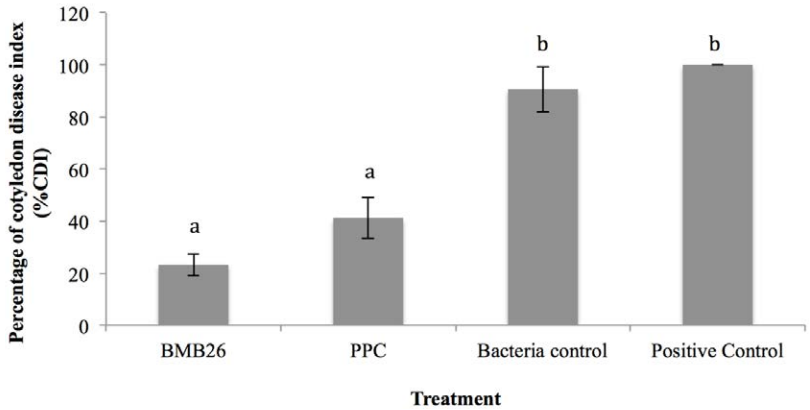

Figure 3: *In vivo* antifungal assay on melon leaves that inoculated with S. rolfsii.

pairs of primers related to lipopeptide gene in *Bacilus*, which are iturin D, bacillomycin C, fengycin D, surfactin A, bacylisin A, and subtilin S were designed and tested to BMB26 genome. PCR amplification of *ituD* (1203 bp), *bacA* (498 bp), *fenD* (269 bp), *srfA* (201 bp) and *bacC* (875 bp) in BMB26 showed positive results, with only the amplification with *subtilin S* did not produce a PCR fragment (Figures 4 and 5).

Discussion

This study has successfully achieved its main aim, which was to find antifungal producing bacteria from bamboo shoot powder that can be used for plant protection. Bamboo has proven to be an excellent plant source for isolating advantageous bacteria strains with beneficial functions. All plants in natural settings establish a mutualistic relationship with microbial community within healthy tissues, thus it is possible to screen and isolate bacterial species from plants that play important role in defending plants from phytopathogens. Previous study showed that bamboo has potential to be rich resource for isolation of beneficial microbes due to its diverse bacteria community, which can reflect the soil microbial abundance [5]. Furthermore, Susanti et al. [4] showed that bamboo rhizosphere has microbial diversity higher than non-bamboo rhizosphere, which related to lower percentage of death in papaya by *Phytophthora palmivora*. The discovery of *B. subtilis* BMB26 in this research provides an evidence of bamboo's potential for further exploration to screen bioactive endophytic bacteria from this resource.

In vitro and *in vivo* assays for BMB26 antifungal compound clearly demonstrated that this isolate has potential to prevent the sclerotium rot disease in melon. When the free-cell supernatant was added to the growth medium, the growth of *S. rolfsii* mycelium was limited and it did not spread through the plate. Similarly, this crude extract also reduced the disease intensity in melon leaves inoculated with the same pathogen by 77% compared to untreated leaves. This antifungal activity of bacteria isolated from plants suggests that they may play a defensive role in plants. The genus *Bacillus* itself represents a group of microorganisms that are widely distributed in most plants and well known for its capability to have a beneficial effect in plants growth and fitness [23]. Formerly, several pest management programs have successfully incorporated the application of some *B. subtilis* strains [24]. Nalisha et al. [25] reported that antifungal compound produced by *B. subtilis* could inhibit 58.3% of *S. rolfsii* growth. Furthermore, Hassen et al. [26] showed that *B. subtilis* G-1 significantly controlled stem rot disease of groundnut and increased the plant growth under greenhouse conditions. Due to BMB26's high potential to replace chemical fungicide, further investigation for ability to control SR under field conditions is required.

The effectiveness of free-cell supernatant indicated that metabolically active cells of BMB26 are not needed for the direct antagonism against *S. rolfsii*, thus it gives an additional advantage that many shortcomings of using live cells biocontrol can be avoided. However, the cell-free supernatant was not equally effective as the live cells for *Pythium sp*. and *Collectotricum sp*., suggesting that for these pathogens the presence of BMB26 live cells is required. This outcome supports the hypothesis of cell interactions, in which for certain antifungal systems, defense mechanisms are activated when microorganisms in close contact [27]. Moreover, antifungal compound

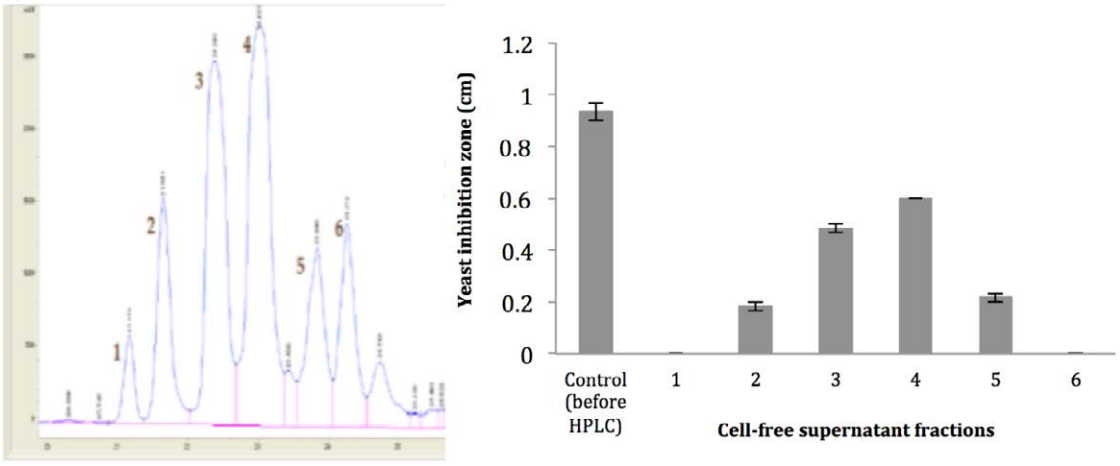

Figure 4: The identification of BMB26 antifungal compound (A) HPLC peaks of BMB26 supernatant after precipitation with HCl and extracted with methanol (B) the antifungal activity of each peak against *Sacharomyces cerevisiae*.

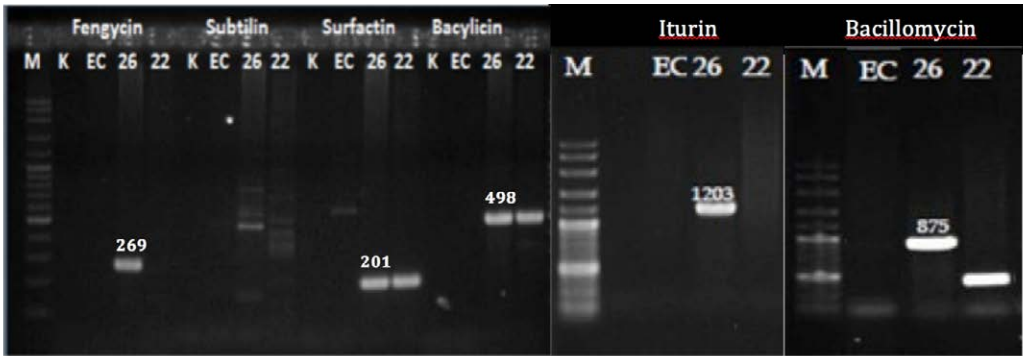

Figure 5: PCR result of BMB26 gene that amplified with pairs of lipopeptide primer, M: marker (1kb ladder), K: negative control, 26: BMB26, 22: Bacillus control.

of BMB26 remained chemically stable in the presence of proteinase-K and at high temperature, which indicated that this compound may not a protein. This result is consistent with finding of Baysal et al. [28], which reported that antimicrobial peptide compounds of *B. subtilis* resistant to high temperature and high concentration of protease.

Due to its inhibitory effect against all pathogen fungi in this experiment, BMB26 may produce a broad antifungal spectrum and release them into the culture medium. Previous study showed that *B. subtilis* produced strong antifungal properties and inhibited growth of different types of plant pathogenic fungi [29]. Stein [11] found that *B. subtilis* produce more than twenty-four structurally diverse antifungal and antibacterial compounds. From the HPLC assay, we found 4 peaks of BMB26 antifungal compound. Using the same extraction method, Grover et al. [18] found 3 different peaks between *B. subtilis* RP24 and its negative mutant. Those 3 active compounds were identified as surfactin, fengycin, and iturin, which are 3 kinds of family of the most lipopeptides produced by Bacillus [30]. It is possible that BMB26 may produce those 3 active compounds because the amplification of its genome showed that it has surfactin, fengycin, and iturin gene. Furthermore, Athukorala et al. [19] showed that majority of *Bacillus* strains produced surfactin and iturin when inoculated against *Sclerotium sp.*, thus strengthen this notion. In addition, BMB26 genome positively contained gene makers of bacillomycin C and dipeptide compound, bacylisin A. Mora et al. [21] found that gene markers for surfactin, bacyllomicin, fengycin, and bacilysin are the most frequent antimicrobial peptide markers for *Bacillus spp*. in natural environment, indicating the important roles of these compounds for the fitness of the strain. Currently, further characterization of these four peaks is being conducted in our laboratory and the result would answer the identity of the antimicrobial peptide compound and the mechanism of BMB26 antifungal compounds.

In addition, this experiment also successfully showed the usage of cotyledon assay as a rapid and accurate technique for the selection of promising biocontrol for *S. rolfsii* under greenhouse condition. This method was modified from Purnamasari et al. [15], which used this assay to identify a SR resistant seedling in *Camelina sativa*. Similar phyllosphere inoculation studies have been widely used for testing the ability of antibiotic producing bacterial to suppress blackleg diseases on *Brassica napus* [31,32]. Interestingly, this assay also showed the induction of resistance in melon inoculated with *S. rolfsii*. Previously, Bargabus et al. [33] used cotyledon assay to observe the induction of induced systemic resistance with *B. mycoides* for the control of Cercospora leaf spot in sugar beet. Similarly, some of the leaves in this experiment showed hypersensitive reaction when inoculated only with BMB26 free-cell supernatant (data not shown), suggesting that this compound may affect resistance gene in plants. Various factors are known to play role in this important plant mechanism, including a result of infection by a pathogen, in response to insect herbivory, upon colonization of the roots by specific beneficial microbes, or after treatment with lipopeptide produced by bacteria [34-36]. Antifungal antibiotics produced by *Bacillus* spp., for example surfactin and fengicyn, are also known to induce resistance characteristic in plant [37,38]. In order to determine if the application of BMB26's lipopetide induces host defence gene expression, further research on plant defense-related chemical and molecular events following treatment with this antifungal compound is required.

Conclusion

This study highlights the potential of bamboo plants to screen antifungal producing bacteria that can be used as a biological control agent. It is clear that *B. subtilis* BMB26, novel bacteria isolated from bamboo powder, produced extracellular products effective against *S. rolfsii*. The melon leaves inoculated with the cell-free supernatant of these bacteria had the least percentage of disease incidences using cotyledon assay. Four peaks of BMB26 antifungal compound were detected and combined with the amplification gene results; it is possible that this strain may produce surfactin, fengycin, and iturin. Furthermore, gene makers of bacillomycin C and dipeptide compound, bacylisin A, are also found in the genome of BMB26. Interestingly, the free-cell supernatant of this strain generated hypersensitive reaction in melon leaves, suggested that besides its antifungal activity, this compound may also play important role to induce the resistance gene in melon. Therefore, the antimicrobial peptide of *B. subtilis* BMB26 has a significant potential as an excellent candidate to replace chemical antifungal for plant protection.

References

1. Le CN, Kruijt M, Raaijmakers JM (2011) Involvement of phenazines and lipopeptides in interactions between *Pseudomonas* species and *Sclerotium rolfsii*, causal agent of stem rot disease on groundnut. J Appl Microbiol 112: 390-403.
2. Kim WG, Cho WD, Jee HJ (1999) Occurrence of Sclerotinia rot on curcubitaceous vegetable crops in greenhouses. Korean J Mycol 27: 198-205.
3. Logan NA, Vos PD (2011) Endospore-forming Soil Bacteria. Springer, Berlin.
4. Susanti WI, Widyastuti R, Wiyono S (2015) Peranan cendawan dan bakteri rhizosfer bambu dalam peningkatan pertumbuhan tanaman dan fenomena disease suppressive soil. Dissertation, Bogor Agricultural University.
5. Han J, Xia D, Li L, Sun L, Yang K, et al. (2009) Diversity of culturable bacteria isolated from root domains of moso bamboo (*Phyllostachys edulis*). Microb Ecol 58: 363-373.
6. Grayston SJ, Campbell CD, Bardgett RD, Mawdsley JL, Clegg CD, et al. (2004) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl Soil Ecol 25: 63-84.
7. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67: 491-502.
8. Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, et al. (2011) The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau, China. Curr Microbiol 62: 182-190.
9. Ashwini N, Srividya S (2014) Potentiality of *Bacillus subtilis* as biocontrol agent for management of anthracnose disease of chilli caused by *Colletotrichum gloeosporioides* OGC1. Biotech: 127-136.
10. Islam MR, Jeong YT, Lee YS, Song CH (2012) Isolation and identification of antifungal compounds from *Bacillus subtilis C9* inhibiting the growth of plant pathogenic fungi. Microbiology 40: 59-66.
11. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56: 845-857.
12. Ongena M, Jacques P (2007) *Bacillus* lipopeptides : versatile weapons for plant disease biocontrol. Trends Microbiol.
13. Wu WJ, Park SM, Ahn BY (2013) Isolation and Characterization of an Antimicrobial Substance from *Bacillus subtilis* BY08 Antagonistic to *Bacillus cereus* and *Listeria monocytogenes*. Food Sci Biotechnol 22: 433-440.
14. Ren JJ, Shi GL, Wang XQ, Liu JG, Wang YN (2013) Identification and characterization of a novel *Bacillus subtilis* strain with potent antifungal activity of a flagellin-like protein. World J Microbiol Biotechnol 29: 2343-2352.
15. Purnamasari M, Cawthray G, Barbetti MJ, Erskine W, Croser JS (2015) Camalexin production in *Camelina sativa* is independent of cotyledon resistance to *Sclerotinia sclerotiorum*. Plant Dis 99: 1544-1549.
16. Sylvester-Bradley R, Makepeace RJ (1984) A code for stages of development in oilseed rape (*Brassica napus* L.). Aspects Appl Biol 6: 399-419.
17. Mohd Zainudin NAI, Abdullah F (2008) Disease suppression in *Ganoderma* infected oil palm seedlings treated with *Trichoderma harzianum.* Plant Prot Sci 44: 101-107.

18. Grover M, Nain L, Singh SB, Saxena AK (2010) Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent *Bacillus subtilis* RP24. Curr Microbiol 60: 99-106.

19. Athukorala SNP, Fernando WGD, Rashid KY (2009) Identification of antifungal antibiotics of Bacillus species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Can J Microbiol 55: 1021-1032.

20. Almoneafy AA, Kakar KU, Nawaz Z, Li B, Ali M, et al. (2014) Tomato plant growth promotion and antibacterial related-mechanisms of four rhizobacterial *Bacillus* strains against *Ralstonia solanacearum.* Symbiosis 63: 59-70.

21. Mora I, Cabrefiga J, Montesinos E (2011) Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int Microbiol 14: 213-223.

22. Dennis P, Edwards EA, Liss SN, Fulthorpe R (2003) Monitoring gene expression in mixed microbial communities by using DNA microarrays. App Env Microbiol 69: 769-778.

23. Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: potential role in developing sustainable systems of crop production. Crit Rev Plant Sci 19: 1-30.

24. Jacobsen BJ, Zidack NK, Larson BJ (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. Phytopathology 94: 1272-1275.

25. Nalisha I, Muskhazli M, Nor Farizan T (2006) Production of bioactive compounds by *Bacillus* subtilis against *Sclerotium rolfsii*. Mal J Microbiol 2: 19-23.

26. Hassen S, Chellappan G, Rethinasamy V (2015) Efficacy of *Bacillus subtilis G-1* in suppression of stem rot caused by *Sclerotium rolfsii* and growth promotion of groundnut. Int J Agric Environ Biotech 8: 111-118.

27. Lacroix C (2011) Protective culture, antimicrobial metabolites and bacteriophages for food and beverage biopreservation. Woodhead Publishing Limited, UK.

28. Baysal Ö, Lai D, Xu HH, Siragusa M, Çalışkan M, et al. (2013) A proteomic approach provides new insights into the control of soil-borne plant pathogens by *Bacillus* Species. *PLoS ONE* 8: e53182.

29. Droby S, Wisniewski M, Macarisin D, Wilson C (2009) Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest. Biol Technol 52: 137-145.

30. Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev.

31. Ramarathnam R, Fernando WG, de Kievit T (2011) The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. BioControl 56: 225-235.

32. Hammoudi O, Salman M, Abuamsha R, Ehlers RU (2012) Effectiveness of bacterial and fungal isolates to control *Phoma lingam* on oilseed rape *Brassica napus*. Am J Plant Sci 3: 773-779.

33. Bargabus RL, Zidack NK, Sherwood JW, Jacobsen BJ (2002) Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol Mol Plant Pathol 61: 289-298.

34. Iavicoli A, Boutet E, Buchala A, Metraux JP (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 16: 851-858.

35. Pang Y, Liu X, Ma Y, Chernin L, Berg G, et al. (2009) Induction of systemic resistance, root colonization and biocontrol activities of the rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones. Eur J Plant Pathol 124: 261-268.

36. Pieterse CMJ, Zamioudis C, Berendsen RL, Van Wees SCM, Bakker P (2014) Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol 52: 347-375.

37. Ongena M, Jacques P, Toure´Y, Destain J, Jabrane A, et al. (2005) Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Appl Microbiol Biotechnol 69: 29-38.

38. Ongena M, Jourdan E, Adam A, Paquot M, Bran A, et al. (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9: 1084-1090.