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Isolation and Identification of Mycorrhizosphere Bacteria and Their Antagonistic Effects Towards *Ganoderma boninense in vitro*

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Basal stem rot caused by *Ganoderma boninense* is the most serious disease of oil palm (*Elaeis guineensis*) in Indonesia and it has caused major loss in palm oil production. Mycorrhizosphere bacteria offer possible advantages as biocontrol agents as they live and proliferate together with arbuscular mycorrhizal fungi, which have an ability to increase plant resistance against pathogens. A study was conducted to isolate mycorrhizosphere bacteria from spores of arbuscular mycorrhizal fungi and test their antagonistic effects against *G. boninense in vitro*. All bacterial isolates were identified based on 16S rDNA analysis and it revealed that eleven out of twenty mycorrhizosphere bacteria isolated were related to *Bacillus* with similarity ranging from 97 to 100%, whereas other isolates were identified as *Pseudomonas*, *Streptomyces*, *Kocuria*, *Enterobacter*, *Brevundimonas,* and *Alcaligenes* with similarities ranging from 96 to 100%. Fourteen out of twenty mycorrhizosphere bacteria showed a varying degree of inhibition towards the growth of *G. boninense in vitro*. Of these, isolate B10 (closely related to *Bacillus subtilis* ZJ06) showed the highest inhibitory effect followed by B17 (closely related to *Bacillus subtilis* N43). Therefore, these bacteria have a potential to be used as biocontrol agents to control basal stem rot disease caused by *G. boninense* in oil palm.

Key words: mycorrhizosphere bacteria, arbuscular mycorrhizal fungi, antagonistic effects, *Ganoderma boninense*

Oil palm (*Elaeis guineensis*) is one of the most important crops in Indonesia. In 2008, Indonesia exported 14.6 million tons of crude palm oil worthing USD 12.659 million (Dharsono 2009). However, some fungal diseases, especially basal stem rot (BSR) disease caused by *Ganoderma boninense* have reduced the annual production of oil palm in South East Asia (Treu 1998). Until now, there is no known cure for BSR and this has led to dramatic yield losses in palm oil production (Sundram *et al*. 2008). Control of BSR through the use of chemicals in the field has not been proven successful (Sapak *et al*. 2008). The use of antagonistic microbes to control the disease as a mean of biological control can be an alternative approach to control the disease.

Arbuscular mycorrhizal fungi (AMF) are known for their beneficial effects on plants including their ability to promote the growth of plants. (Blal *et al*. 1990). The ability of AMF to promote plant growth can be attributed to protection against root pathogens and production of substances affecting plant growth (Phosri *et al*. 2010). Hashim (2004) reported that inoculation of oil palm seedlings with AMF suppressed BSR disease incidence and prolonged incubation time required for *Ganoderma* to cause infection in the seedlings.

Arbuscular mycorrhizal fungi coexist with other soil organisms in the rhizosphere (Bharadwaj *et al*. 2008). Bacteria associated with AMF are defined as mycorrhizosphere bacteria (Artursson *et al*. 2006). Garbaye (1991) defined mycorrhizosphere as a rhizosphere of a root infected by a mycorrhizal fungus. Garbaye stated that as the fungus uses some of root exudates and modifies root functions, microbial communities in the mycorrhizosphere differ from those in the rhizosphere and in the soil. It is considered that changes in microbial populations in the mycorrhizosphere could be involved in plant disease suppression. The mechanisms involved in disease suppresion could be mainly due to induction of the host defence mechanisms such as the formation of structural barriers, lignification of cell walls, and the production of antifungal metabolites to slow down the progress of infection and improve plant growth and vigor (Sapak *et al*. 2008).

Isolation and identification mycorrhizosphere bacteria from rhizosphere of uninfected oil palm surrounded by oil palm heavily infected by *G. boninense* could be useful as a preliminary study of biocontrol of BSR in oil palm. We assumed that the absence of *Ganoderma* infection in the plant that is surrounded by infected plants can be attributed to the presence of microbes in the rhizosphere of the plant that trigger resistance to *Ganoderma* attack. Thus, this region could be a good source of mycorrhizosphere bacteria. The objectives of this study were to isolate and identify mycorrhizosphere bacteria from spores of AMF in the rhizosphere of oil palm that showed antagonistic effects against *G. boninense*.

MATERIALS AND METHODS

Isolation of Arbuscular Mycorrhiza Fungal Spores. Spores of AMF were isolated from rhizosphere of oil palm plantings in the Aek Pancur Plantation belonging to the Indonesian Oil Palm Research Institute, North Sumatera, during September 2006. Soil samples were analysed to determine nutrient contents at the Soil Research Institute, Bogor. Spores of AMF were isolated from 500 g of soil samples using a decanting-sieving-method (Brundrett *et al*. 1994). Spores were counted using a dissecting microscope and the spores were kept for later use for bacterial isolation.

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Isolation of Mycorrhizosphere Bacteria. Mycorrhizosphere bacteria were isolated from spores of AMF using a method described by Reimann (2005). Collected spores were crushed using sterile needles and transferred to Petri dishes containing nutrient agar (NA) with concentrations of 1×, 10×, and 100×; tryptic soy agar (TSA) with concentrations of 1×, 10×, 100×; and *Pseudomonas* agar base (PAB) at 1× concentration. All the crushed spores were put on the surface of the agar media and then incubated for 48 h at 20°C. All bacteria that grew on the media were transferred to another agar media until single colonies were isolated. All single colonies were transferred to agar slants and stored at 4°C. Morphological examination was then conducted on these colonies.

DNA Extraction. Total DNA of the bacteria was extracted using an Instagene Matrix Kit (BioRad). One-day-old bacterial colonies on slant agar were added with 1.0 mL of sterile water in order to get suspension of bacteria. The bacterial suspension was transferred to 1.5 mL eppendorf tubes, suspended with sterile water and centrifuged at 10 000 × g for 1 min. The supernatants were decanted and the pellets were resuspended with 200 µL Instagene Matrix. The bacterial suspension was then incubated at 56°C for 15-30 min on a heat block, vortexed with high speed for 10 sec and returned to the heat block, incubated at 100°C for 8 min, and vortexed again with high speed for 10 sec, then repelleted at 10 000 × g for 2-3 sec. The supernatant containing the DNA was then stored at -20°C.

Polymerase Chain Reaction. Five µL of DNA template was mixed with 45 µL PCR solution containing 5 µL of 25 mM $MgCl_2$, 4 µL of 2.5 mM dNTP mix, 5 µL of 10x PCR buffer, 0.25 µL LA tag, 2 µL of 10 M Primer 8F, 2 µL of 10 M Primer 1492 R, and 26.75 µL ddH_2O in a total final reaction volume of 50 µL. Amplification of the peptide synthetase was carried out using the universal primers 8F (5' GGTTACCTTGTTACGACTT 3') and 1492R (5' AGAGTTTGATCCTGGCTCAG 3') from AlphaDNA (Canada). PCR was carried out as follows: initial denaturation at 96°C for 3 min, 30 cycles consisting of denaturation at 96°C for 45 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 2 min. The reaction was completed by a final extension at 72°C for 7 min. PCR products were then analyzed by electrophoresis in a 0.8% (w/v) agarose gels in 1x TAE running buffer at 100 V for 30 min. Gels were visualized under a UV illuminator and visible DNA bands that corresponded to the expected size were cut and purified using a Gene Aid Kit.

DNA Sequencing. The primers used for cycle sequencing were 765R (5' CTGTTTGCTCCCCACGTTTC 3') and 1141R (5' GGGTTGCGCTCGTTGC 3') from AlphaDNA (Canada). Cycle sequencing solution contains the following components: 2 µL of 5x sequencing buffer, 2 µL primer mix (765 R and 1141R), 4 µL big dye V3.1, 4 µL DNA template and 8 µL H_2O to give a final reaction mixture of 20 µL. The cycle sequencing was performed as follows: initial denaturation at 96°C for 3 min, 25 reaction cycles

The cycle sequencing products were then purified prior to reading the DNA sequence. The resulting DNA from cycle sequencing was first purified by precipitating the DNA using ethanol, sodium acetate, and EDTA followed by centrifugation and rinsing the pelllets with 70% ethanol. Purified DNA was resuspended in 12 µL of ddH_2O prior to reading the DNA sequence using Genetic Analyzer 3130 (Applied Biosystems, USA). The DNA contigs were assembled using ATGC program that connects the 765R and 1141R primers. The resulting sequences were compared with DNA sequences available in the GenBank database of NCBI using BLAST program.

Phylogenetic Tree Construction. Alignment of 16S rDNA sequences was performed using ClustalX. The phylogenetic tree was constructed by comparing 16S rDNA sequences of twenty isolated mycorrhizosphere bacteria with 16S rDNA sequences of twenty bacteria from GeneBank DNA database and then visualized using Tree View 1.6.6. The phylogenetic relationship was revealed by neighbor-joining analysis combined with bootstrap analysis from 100 replicates.

Antagonistic Effects of Mycorrhizosphere Bacteria towards *Ganoderma boninense*. All mycorrhizosphere bacteria were screened *in vitro* for their ability to inhibit the growth of *G. boninense* by measuring area of clearing zones formed surrounding the bacterial colonies. One-mL-aliquots of each bacterial suspension was inoculated into 50 mL nutrient broth, tryptic soy broth and *Pseudomonas* broth. The bacterial cultures were incubated in a shaking incubator (150 rpm) at 28°C for 12 h.

Isolates of *G. boninense* were inoculated onto potato dextrose agar (PDA) and spread all over the agar surface using sterile cotton buds. Four sterile paper discs (6.0 mm diameter) were used for antagonistic activity test. Each paper disc was dipped with bacterial suspension, placed on the agar surface, and then put in a refrigerator at 4°C for 1.5 h to allow absorbtion of the bacterial suspension into the media. The cultures were then incubated at 28°C for three days. The antagonistic effects of the bacteria were evaluated by measuring the area of the clearing zones.

A completely randomized design with two factors was used to analyze the data. The first factor was the type of bacterial inoculum. The second factor was the volume of bacterial suspension inoculated onto the media. Discs that dipped in media without bacteria was defined as a negative control, while inoculation with antifungal chloramin-T 2% was used as a positive control. Each treatment was replicated 4 times.

RESULTS

Isolation and Identification of Mycorrhizosphere Bacteria. A total of 56 mycorrhizosphere bacteria were isolated from spores of AMF. There were 24 isolates that grew on NA, 29 isolates on TSA, and 3 isolates on PAB.

Colonies of the mycorrhizosphere bacteria were selected based on their morphological characteristics such as color and shape of the colonies. The results showed that from 56 isolates of mycorrhizosphere bacteria, there were 20 isolates that showed different morphological characteristics (Table 1). All of the 20 mycorrhizosphere bacteria were then identified based on their 16S rDNA. Phylogenetic analysis of 16S rDNA showed that the mycorrhizosphere bacteria were dominated by *Bacillus* spp. (11 out of 20 isolates: B2, B4, B5, B8, B10, B11, B13, B14, B15, B16, and B17). The other groups belonged to the genera *Streptomyces* (B1), *Enterobacter* (B6), *Brevundimonas* (B9), *Kocuria* (B3), *Alcaligenes* (B7, B12, and B18) and *Pseudomonas* (B19 and B20) (Table 2). Curiously, even though isolate B14 and B16 were closely related to *Bacillus thuringiensis* AE1.16 with 100% similarity, the color of their colonies was very different on NA media at the same age (Fig 1).

Antagonistic Effects of Mycorrhizosphere Bacteria against *Ganoderma boninense*. Among the 20 isolates of mycorrhizosphere bacteria, 14 isolates (B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B14, B16, B17, and B19) showed an ability to inhibit the growth of *G. boninense in vitro* as indicated by clearing zones formed around the bacterial colonies (Fig 3). Isolate B10 (closely related to *Bacillus subtilis* ZJ06) gave the largest clearing zone (52.84 mm^2) followed by B17 isolate (closely related to *B. subtilis* N43) with clearing zone area of 44.80 mm^2. In contrast, six other mycorrhizosphere bacteria isolates (B1, B2, B13, B15, B18, and B20) did not exhibit any inhibition to the growth of *G. boninense*.

Table 1 Morphological characteristics of selected mycorrhizosphere bacteria isolated from spores of arbuscular mycorrhizal fungi

Isolate code	Media and concentration	Color and form of colonies on agar plate
B1	NA 1x	Light brown, undulate
B2	TSA 1/10x	White, undulate
B3	NA 1x	Yellow, entire (smooth)
B4	NA 1x	Yellow-cream, filiform
B5	NA 1/10x	White, filiform
B6	TSA 1/10x	Creamy-white, entire
B7	TSA 1x	Cream-brownish, lobate
B8	NA 1x	Creamy-white, entire
B9	NA 1/100 x	Pink, entire
B10	TSA 1x	Creamy-white, lobate
B11	NA 1x	White, entire
B12	TSA 1x	Cream, lobate
B13	TSA 1x	Brown, lobate
B14	TSA 1x	Yellow-cream, lobate
B15	NA 1x	Creamy-white, entire
B16	TSA 1/10x	Red-white, undulate
B17	NA 1x	White, filiform
B18	TSA 1x	Brown, undulate
B19	PAB 1x	Orange to bright red-yellow, entire
B20	PAB 1x	Orange to bright red-yellow, entire

Table 2 Identification of selected mycorrhizosphere bacteria based on their 16S rDNA.

Isolate code	Bacterial strains	Accession number	Homology (%)
B1	*Streptomyces* sp. FXJ1.297	FJ754492.1	96
B2	*Bacillus* sp. TDSAS2-16	GQ284549.1	98
B3	*Kocuria* sp. 10-4DEP	GQ203109.1	99
B4	*Bacillus subtilis* N43	GQ465935.1	97
B5	*Bacillus thuringiensis* CCM11B	FN433030.1	100
B6	*Enterobacter* sp. JS-48	GQ280118.1	97
B7	*Alcaligenes faecalis* AE1.16	GQ284565.1	100
B8	*Bacillus pumilus CrK08*	GQ503326.1	100
B9	*Brevundimonas* sp. ZF 12	GQ891673.1	100
B10	*Bacillus subtilis* ZJ06	EU266071.1	100
B11	*Bacillus clausii* CSB15	FJ189790.1	99
B12	*Alcaligenes faecalis* AE1.16	GQ284565.1	100
B13	*Bacillus* sp. AHE.1	AY485275.1	99
B14	*Bacillus thuringiensis* CCM11B	FN433030.1	100
B15	*Bacillus pumilus* CrK08	GQ503326.1	100
B16	*Bacillus thuringiensis* CCM11B	FN433030.1	100
B17	*Bacillus subtilis* N43	GQ465935.1	97
B18	*Alcaligenes* sp. F78	EU443097.1	98
B19	*Pseudomonas stutzeri* TSWCW11	GQ284458.1	99
B20	*Pseudomonas stutzeri* TSWCW19	GQ284464.1	100

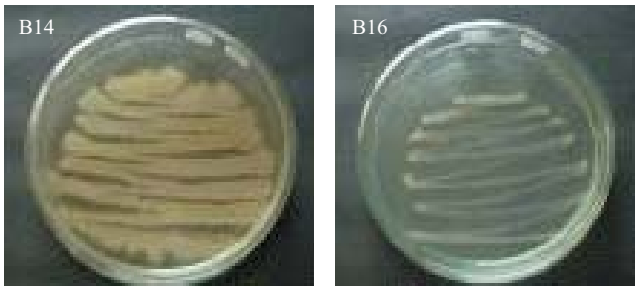

Fig 1 Two isolates of mycorrhizosphere bacteria (B14 and B16) were closely related to *Bacillus thuringiensis* AE1.16 with 100% similarity but their colonies grown on nutrient agar were different at the same age.

DISCUSSION

The present study showed a possibility that mycorrhizosphere bacteria isolated from spores of AMF can be exploited as biological control agents towards *G. boninense*. *Bacillus* spp. and related genera, which are common soil bacteria, have been identified as potential biological control agents towards pathogens (Ferreira *et al*. 1991). In this study, the majority (11 out of 20 isolates) of the isolated mycorrhizosphere bacteria were *Bacillus* sp. This finding was in line with Xavier and Germida (2003) who found that around 80 to 92% of bacteria isolated from *Glomus clarum* NT4 spores were endospore-forming *Bacillus* spp. Mahaffee and Kloepper (1997) also reported that *Bacillus* sp. is the dominant bacteria found in the rhizosphere due to their versatile physiological ability with

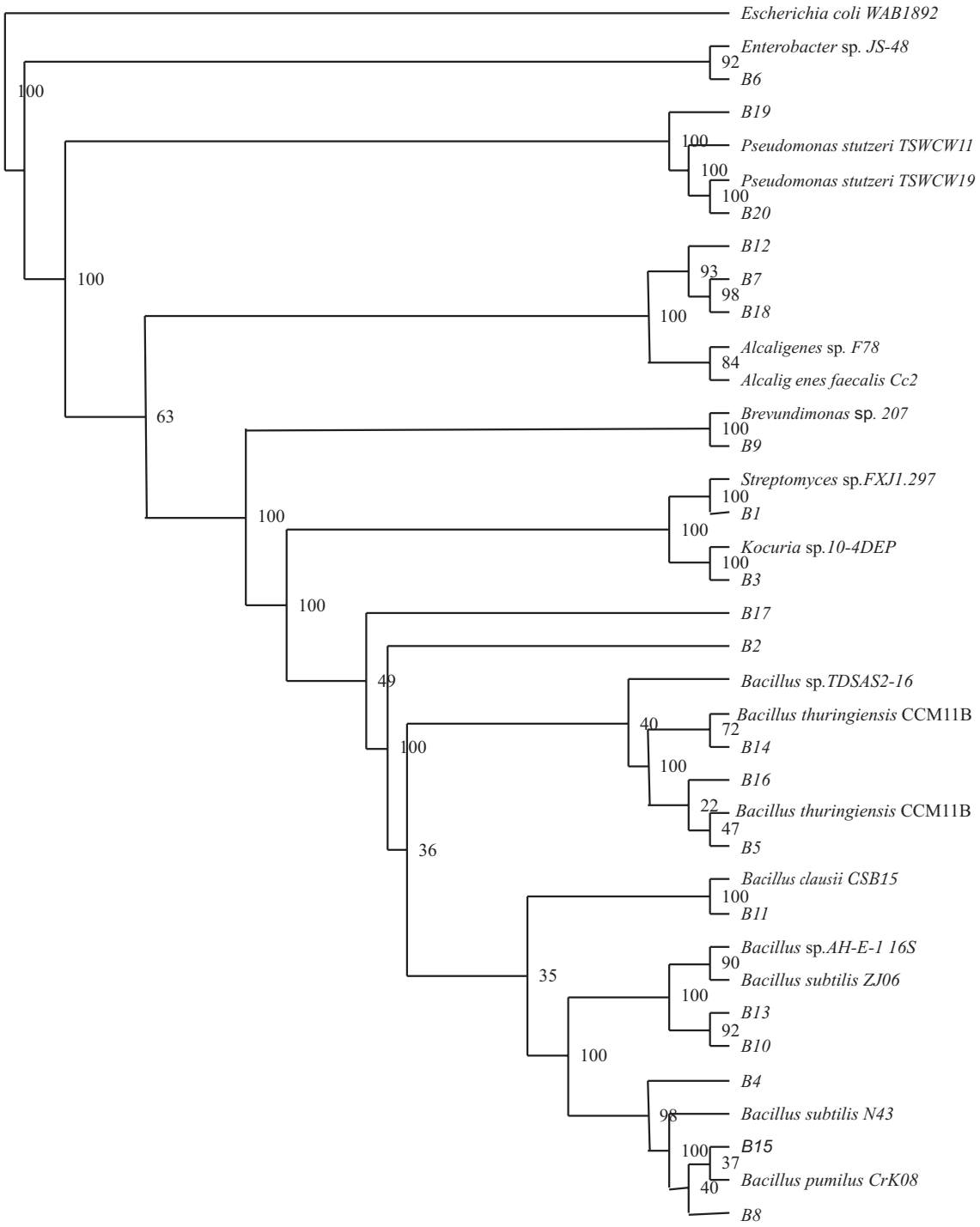

Fig 2 Dendrogram of selected mycorrhizosphere bacteria isolated from spores of arbuscular mycorrhizal fungi in the rhizosphere of oil palm. Eleven out of twenty isolates belong to the genera of *Bacillus*.

respect to heat, acidity and salinity tolerance. In this study, most of the mycorrhizosphere bacteria, including *Bacillus* sp. were Gram positive. Artursson *et al*. (2005) reported that Gram positive bacteria may be more commonly associated with AMF than Gram negative bacteria. Andrade *et al.* (1997) also reported that genera *Arthrobacter* and *Bacillus* were most frequent in the hyposphere, the area of soil surrounding individual AMF hyphae.

In this study, we found some isolates that are homologous based on their 16S rDNA showed different color on NA medium at the same age. The identification of bacteria based on 16S rDNA is effective to characterize the relationship between species. We assume that these isolates are located in different sub-species but need further studies to prove it such as using DNA markers at different loci of ribosomal DNA regions. Fox *et al*. (1992) stated that effective identity of 16S rRNA sequences is not necessarily a sufficient criterion to guarantee species identity. Thus, even though 16S rRNA sequences can be used routinely to differentiate and find out relationships between genera and

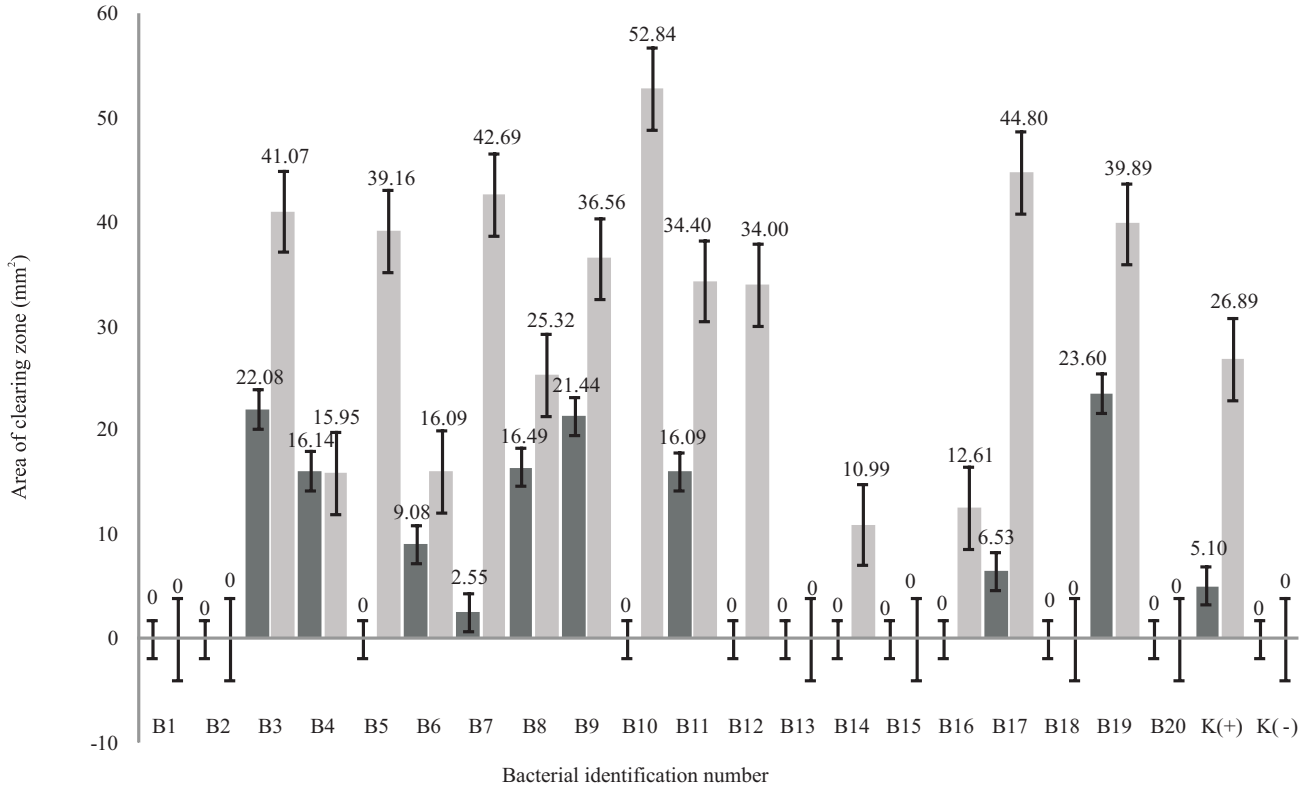

Fig 3 Area of clearing zone as an indication of inhibitory effects of mycorrhizosphere bacteria against the growth of *Ganoderma boninense in vitro*. Chloramin-T 2% (w/v)was used as positive control K(+) whereas liquid medium without bacterial cells was used as negative control K(-). B10 isolate gave the largest clearing zone (52.84 mm^2) followed by B17 isolate (44.80 mm^2). ■, 10 µL of bacterial suspension. ■, 20 µL of bacterial suspension.

well-resolved species, very recently different species may not be recognizable.

The appropriateness of nutrients in nutrient-rich media such as NA and TSA to support the growth of mycorrhizosphere bacteria was investigated. Both media are known to support the growth of a large variety of bacteria. Some bacteria might require smaller amount of nutrients than others. Thus, in this study, different concentrations of the media resulted in variations on the growth of bacteria. *Pseudomonas* agar base is known as a selective medium and it only encourages *Pseudomonas* bacterial growth on the medium. There were only two mycorrhizosphere bacteria that grew on this medium.

Two isolates of mycorrhizosphere bacteria B10 and B17 showed the highest antagonistic effects against pathogenic fungi *G. boninense in vitro* as indicated by the most prominent clearing zones observed around colonies of these bacteria. Budi *et al*. (1999) found that 12.5% of all bacteria isolated from mycorrhizosphere showed antagonistic effects towards various soilborne pathogens *in vitro*. They also suggested that mycorrhizosphere may be rich in plant health promoting rhizobacteria (PHPR). Among PHPR, *Pseudomonas* spp. and *Bacillus* spp. appear to have the greatest potential as biocontrol agents because they are dominant in the rhizosphere and have the ability to compete with pathogens to improve plant health (Mahaffee and Kloepper 1997). Biocontrol activity occuring in soils with AMF is linked to associated bacteria (Cruz *et al*. 2008). There is a large number of publications that reported the potential use of *B. subtilis* as biocontrol agents against pathogenic fungi and bacteria (Ferreira *et al*. 1991; Leelasuphakul *et al*. 2008; Velmurugan *et al*. 2009). A study by Nalisha *et al*. (2006) found that *B. subtilis* produced bioactive compounds that are active as antifungal agents against plant pathogenic fungi, *Sclerotium rolfsii*. A number of *B. subtilis* strains suppressed the growth of several pathogenic fungi by producing antifungal substances such as iturin A, a group of similar cyclic lipopeptides (Gong *et al*. 2006); surfactin (Souto *et al*. 2004); and amicoumacin (Pinchuk *et al*. 2002).

Bacillus spp. are appealing candidates for biocontrol agents because they produce endospores that are tolerant to heat and desiccation (Weller 1988). Choudary *et al*. (2009) stated that certain strains of spore-forming *Bacillus* spp. can elicit induced systemic resistance (ISR) that results in resistance to a broad range of pathogens. Other bacteria such as *Enterobacter*, *Streptomyces* have been shown to effectively control diseases caused by soilborne pathogens (Chernin *et al*. 1995; Singh *et al*. 1999). In this study, isolate B10 (*B. subtilis* ZJ06) has a potential to be used as a biocontrol agent against *G. boninense*. There are several possible mechanisms underlying the antagonistic effects of these bacteria towards the growth of *G. boninense*. Such mechanisms are production of antifungal compounds, antagonism through antibiosis and cell wall breakdown by

cell-wall-degrading enzymes. It still requires further investigations to determine the mechanism involved in inhibition of *G. boninense* by these bacteria. The production of antifungal compound pyrrolnitrin produced by *Pseudomonas* showed an antagonistic effect against *Rhizoctonia solani* (Ligon *et al*. 2000). Siderophores that transport irons into bacterial cells are able to sequester iron (III), thus reducing iron availability to the pathogen (Yang and Crowley 2000). Some bacteria are able to produce lytic enzymes that can lead to degradation of pathogen cell walls (Singh *et al*. 1999). The basis of antibiosis as a biocontrol mechanism of PHPR has been increasingly better understood over the past two decades. Several studies found a variety of antibiotics that have been identified, including compounds such as oligomycin A, kanosamine, zwittermicin A and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. that were acted as biocontrol agents towards plant pathogenic fungi (Milner *et al*. 1995; Milner *et al*. 1996; Nakayama *et al*. 1999; Kim *et al*. 1999; Raaijmakers 2002). Further study is required to investigate the mechanisms of antagonistic of the mycorrhizosphere isolates towards *G. boninense*.

Combination of AMF and mycorrhizosphere bacteria with different mode of actions might strengthen the efficacy of these potential biocontrol agents towards pathogenic fungi rather than a single biocontrol agent. Whether AMF could be used as biocontrol agents practically, or possibly function as vectors for associated bacteria with biocontrol properties, remains to be explored. Based on the findings of this study, it can be concluded that mycorrhizosphere bacteria isolated from AMF spores can be used as antagonistic agents towards pathogenic fungi *G. boninense,* a causal agent of BSR disease in oil palm. Further study is needed to investigate possible mechanisms involved in the antagonism of the mycorrhizosphere bacteria towards the growth of *G. boninense in vitro* and *in vivo*.

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