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DIAZOTROPHIC BACTERIA AS BIOLOGICAL CONTROL AGENT FOR *Lasiodiplodia theobromae*ISOLATED FROM KENAF SEEDS

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ABSTRACT

Lasiodoplodia theobromae may cause deterioration and loss of germination in seeds. This study has identified *L. theobromae* as a causal agent of black seed rot and causes germination loss up to 95% in infected kenaf seeds. Preliminary screening was done to evaluate the antagonistic effects of selected diazotrophic bacteria on *L. theobromae* by dual culture assay (in vitro). This study was conducted to control the seed-borne pathogen, *L. theobromae* with antagonistic bacteria as biological control agent. There were 3 species of diazotrophic bacteria in biological seed treatment namely *Burkholderia cepacia*, *Burkholderia vietnamiensis* and *Bacillus subtilis*. In vivo experiment was done to evaluate the efficacy of selected antagonistic bacteria against *L. theobromae*. The results showed that *B.cepacia* has a significant potential as biological control agent to suppress the growth of seed-borne pathogen, *L. theobromae* by indicating on the increased seed germination.

Keywords:diazotrophic bacteria, biological control, *Hibiscus cannabinus* L., seed-borne pathogen.

INTRODUCTION

Kenaf (*Hibiscus cannabinus*L.) is a common warm season annual fibre plant of tropical and subtropical Africa and Asia.Product that has been successfullyproduced from kenaf plant is papers, rope, twine, bag, rugs and many more.In Malaysia, kenaf has been cultivated as a substitute crop for tobacco. Although kenaf is tolerant towards many planting condition, however, previous studies was reported that kenaf can be infected by phytopathogens including fungi, bacteria and nematodes (Mat Daham *et al*., 2005).

Many potential pathogens have reported that may cause disease infection on kenaf plant such as *Rhizoctonia solani*, *Sclerotium rolfsii* (Swart and Tesfaendrias, 2003), *Phytophthora parasitica*,*Ralstonia solanacearum*(Mat Daham *et al*., 2005) and nematodes (*Meloidogyne incognita*) (Lawrence and McLean, 1992). *Lasiodiplodia theobromae* was reported as seed-borne pathogen in other plants includes maize seeds (Owolade *et al*., 2001) and bitter gourd (Sultana and Ghaffar, 2009).

Seed-borne pathogen is one major cause for loss in seed germination. Several chemical pesticides including carbofuran, fipronil, isoprocarb and carbaryl have been applied on the seedling, roots and leaves to control disease infection (Mat Daham *et al*.,2005).Laboratory evaluation of seed treatment of rice with Carbendazim (Barvistin) and thiram were effectively controlled seed rot disease and significantly decreased seed motility (Narmada and Kang, 1992). However,highly dependence on chemical treatment may lead to excessive application which cause deterioration in soil system and affect other beneficial microorganism's survival. Concern related to harmful effect of chemical pesticides has switched the attention to other alternative method which is biological control agent including fungi and bacteria.

Diazotrophic bacteria have a broad potential as a biocontrol agents against seed-borne fungal pathogens to increase germination rate of kenaf seeds and as a part of disease management in plant infection. Diazotrophic bacteria is a group of microorganisms including bacteria and Archea that can be found in the rhizosphere soil of plants (Döbereiner, 1992) and involved in biological nitrogen fixation (BNF) process; due to its ability to convert atmospheric nitrogen to ammonia which can be used to promote plant growth and yield.

Previous study stated that diazotrophic bacteria were isolated from rhizosphere area of various crop parts including *Azotobacter, Pseudomonas, Burkholderia* and *Herbaspirillum, Arthrobacter,Azoarcus, Bacillus, Beijerinckia, Dertix, Enterobacter, Klebsilla, and Zooglea* (Barraquio *et al*., 2005). *Burkholderia* spp. was found colonizing the cortex cells, endodermis, xylem and between pit cells that can prevent multiplication of vascular pathogens and reduce disease incidence (Chen *et al*., 1995). This study worked on isolation and identification of antagonistic bacteria from kenaf rhizosphere and their potential against seed-borne pathogen, *L. theobromae*.

MATERIALS AND METHODS

Isolation of diazotrophic bacteria from kenaf roots

Five replicates of root samples were taken from kenaf cultivation area in Kg. Baru Panggas and Felda Chuping, Perlis. Root samples were collected and stored in cool box for transportation. Samples were weighed, cut into small pieces and mix (1:9) in sterile distilledwater. Samples were homogenized and shaken at 200 rpm for 30 min. Serial dilution was prepared and 0.1 ml aliquots of each dilutions were spread on nutrient agar and then sub-cultured on nitrogen free agar.Pure colonies with different morphological appearanceson the medium were isolated and sub-cultured again for further analysis. The pure culture was stored in 80% glycerol at -20^{o}C.

Establishment of antagonism activity in vitro

The ability of diazotrophic bacteria as biological control agent was evaluated in vitro using dual culture assay experiment. The radius of *L. theobromae* colony growth towards the bacterial streaks was measured and percentage of fungal growth inhibition was calculated using the formula: A - B/A x 100, where A is the distance of mycelia growth away from the bacterium, and B is the distance of mycelia growth toward the bacterium (Sfalanga *et al*., 1999).

DNA extraction, 16S rRNA gene amplification and sequencing of bacterial isolates

Genomic DNA was extracted by using simple boiling method. 16S rRNA gene was amplified by PCR with universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Turner *et al*., 1999). The oligonucleotides were synthesized by 1st Base Sdn. Bhd. PCR reaction was carried out in a 25 µl reaction volume. Each PCR reaction contained 3 µl of DNA template, 0.5 µL of 10mM dNTP, 0.6 µl of 25 mM $MgCl_2$, 0.2 µl of 10x Taq polymerase buffer, 0.5 µl of each primer (10 mM) and 0.2 µl (0.5 units) Taq DNA polymerase (Fermentas, Inc.). DNA amplification was done in a Bio-Rad Thermoblock with the following procedure: an initial denaturation at 95°C for 3 min, 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 57°C) and extension (1 min for 72°C); followed by the final extension at 72°C for 10 min. Five µl of each amplified PCR product was run on a 2% agarose gel in 1X TAE buffer. DNA bands were detected by ethidium bromide staining and visualized by UV light transluminator. PCR products were purified by using GeneJet PCR Product Purification Kit (Fermentas, USA) and submitted for sequencing. Nucleotide sequences were identified by using Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed by using Neighbor-Joining method with default values with MEGA software version 4.

Efficacy of antagonistic bacteria against inoculated kenaf seeds

Kenaf seeds were surface sterilized using 70% ethanol, 5% sodium hypochlorite and rinsed two times with sterile distilled water. Eighteen seeds were inoculated in mycelia suspension and shake for 30 min using orbital shaker. Bacterial suspension was prepared from three different isolate with concentration of 10^9 CFU per ml. The inoculated seeds were dipped into prepared antagonist bacterial suspension. After 30 min, the seeds were taken out from the suspension, placed on sterile filter paper and dried under laminar air flow cabinet to release excess bacterial suspension. Treated seeds were placed in pot containing sterile soils and incubated for germination. Germinated seed was recorded at 3 days interval. Each treatment was replicated 6 times.

Experimental design and statistical analysis

All the experiments were conducted in Completely Randomized Design (CRD) and data collection was subjected to analysis of variance and means were compared using Duncan's Multiple Range Test.

RESULT

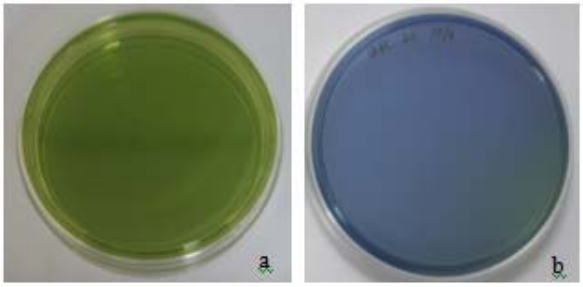

Figure-1. Color changes by diazotrophic bacteria grow onN-free medium at 72 h after incubation period. (a) Control (nobacteria); (b) Inoculated plate.

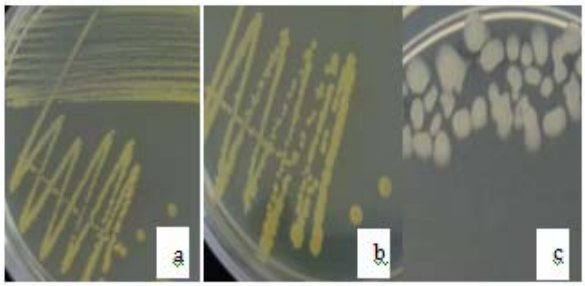

Figure-2. Diazotrophic bacteria grow on NA medium at 48 h after incubation. (a) RKBP3d; (b) RFC1a; (c) IRPP1b.

In vitro antagonistic tests

Dual culture assay was done to determine the antagonist potential of diazotrophic bacteria to suppress *L. theobromae* growth.Obvious inhibition zone of *L. theobromae* mycelia indicates that the selected diazotrophic bacteria have the capability to block the mycelia penetration. There was a free zone area between bacterial isolates and fungal culture due to this inhibition activity. Among 68 bacterial strains isolated from rhizosphere of kenaf roots, three strains were exhibited potent antagonistic effect against *L. theobromae* on PDA (Figure-3). Percentage inhibition radial growth (PIRG) values were showed significantly difference ($P<0.05$) on antagonism activity on fungal pathogen. Three bacterial isolates gave PIRG value more than 60% on *L. theobromae*. The highest PIRG value was showed by IRPP1b. Another 2 isolates were RFC1a and RKBP3d.

After 3 days of incubation, RKBP3d, RFC1a and IRPP1b had established contact with mycelia growth of *L. theobromae* in vitro. The result recorded an inhibition rangeis between 0.2 cm to 6.0 cm as shown in Figure-4.

However, there is no significant difference on average diameter mycelia inhibition for all the bacterialisolates. ANOVA revealed that these 3 bacterial isolates have same effect on mycelia growth inhibition.

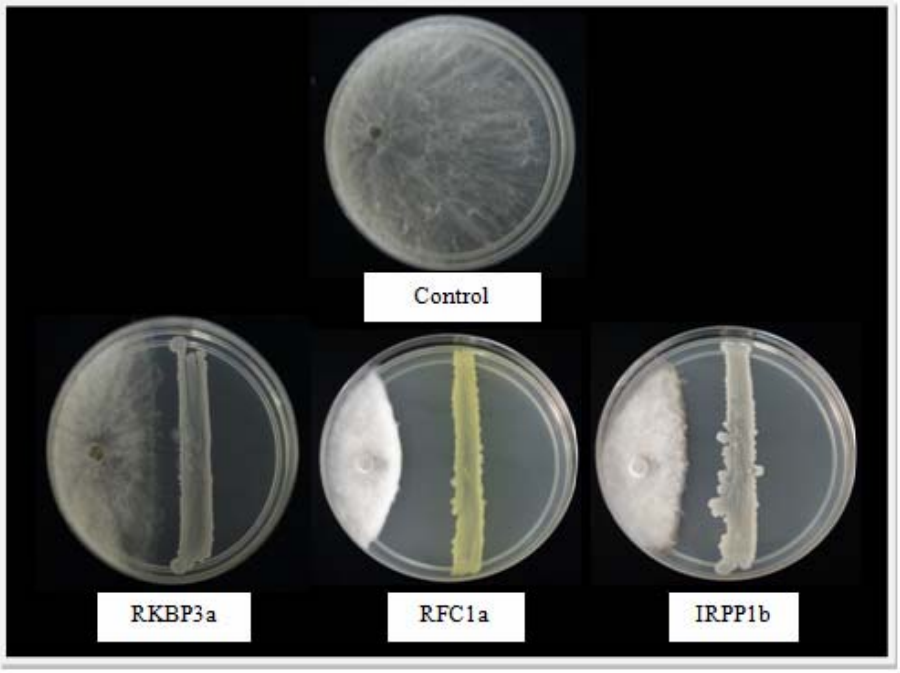

Figure-3. Dual culture between bacterial isolates and L. *theobromae* on PDA at 7 days after incubation.

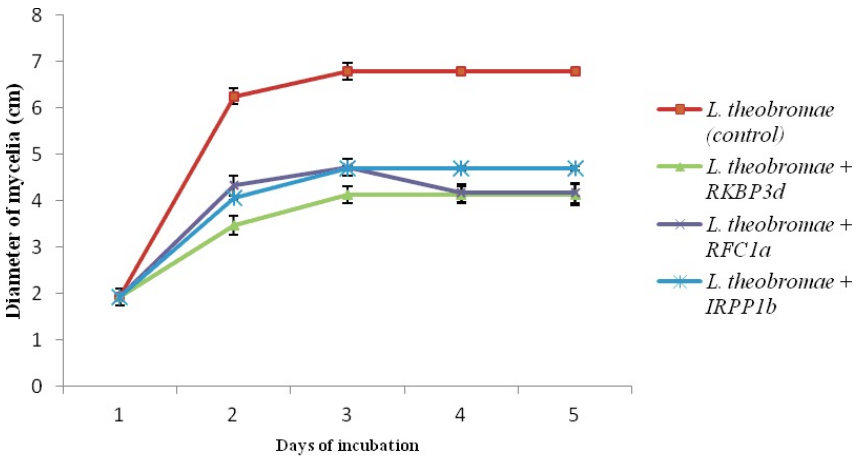

Figure-4. Radial mycelial growth of *L. theobromae* on PDA medium inoculated with diazotrophic bacteria.

16S rDNA sequence analysis

The 16S rDNA sequence similarity of isolate RKBP3d was 99% to *Burkholderia cepacia*. Isolate RFC1a was observed to have 100% similarity with *Burkholderia vietnamiensis*. The closest neighbor to isolate IRPP1b was *Bacillus subtilis* with 100% similarity. The sequences obtained from these isolates were deposited in NCBI with the accession numbers as followed, isolate RFC1a: JF833110, isolate RKBP3D: JF820823 and isolate IRPP1b: JF8331. In this present study, 3 bacterial species isolated from kenaf rhizosphere have been identified and described. Molecular approach using 16S rDNA markers analysis confirmed the bacterial species were *B. cepacia*, *B. vietnamiensis* and *B. subtilis*. Molecular approach using 16S rDNA markers analysis confirmed the bacterial species were *B. cepacia*, *B. vietnamiensis* and *B. subtilis*.

Referring to below phenogram, on the bases of a Neighbor-Joining (NJ) analysis with 1000 boostrap replications, all isolates were divided into 2 major clusters (a) and (b) (Figure-5). Major cluster (a) was belong to *Bacillus* genera. Group (a) consisted of *Bacillus subtilis* [a(i)] and *Bacillus megaterium* [a(ii)].

One of the antagonist bacteria (IRPP1b) was grouped in group [a(i)] same group with *B. subtilis* strain BCRC17435 (EF423598.1). Meanwhile, major cluster (b) isolate belong to *Burkholderia* group. Another two bacterial isolates (RFC1a and RKBP3d) were grouped in the subgroup [b(ii) 1]. Isolate RFC1a was same with *B. vietnamiensis*strain RPB3 (HQ606073.1) and RKBP3d similar to *B. cepacia* strain RFW-8 (HQ650586.1). The resultant NJ dendogram from this analysis were well-fitted in this group with maximum identity 99% and 100%.

Table-1. Identified diazotrophic bacteria from 16S rRNA gene comparison with sequencedata from Genebank.

Isolate	Closest neighbour	%Similarity	Accession number
RKBP3d	*Bukholderia cepacia* strain RFW-8 (HQ650586.1)	99%	JF820823
RFC1a	*Burkholderia vietnamiensis* strain RPB3 (HQ606073.1)	100%	JF833110
IRPP1b	*Bacillus subtilis* strain BCRC17435 (EF423598.1)	100%	JF833115

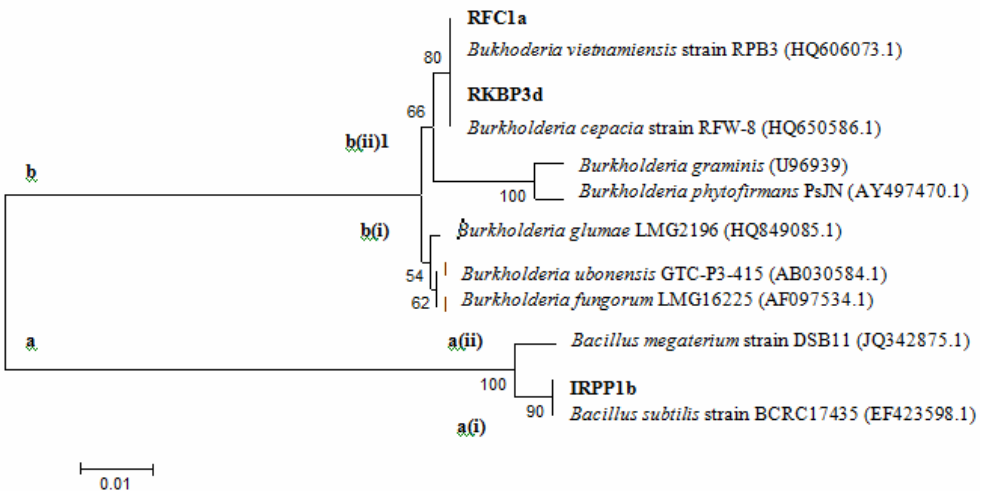

Figure-5. Neighbor-Joining tree based on 16S rRNA gene sequence similarity showing the phylogenetic position of the selected diazotrophic bacteria strains RKBP 3d, RFC1a and IRPP1b obtained from kenaf roots. Boostrap analysis was made with 1000 cycles. Bar scale, 0.1 substitutes per nucleotide position.

Efficacy of antagonistic bacteria against inoculated

Three species of selected antagonistic bacteria were *B. cepacia*, *B. vietnamiensis* and *B. subtilis* that have been used in seed treatment of inoculated un-germinated kenaf seeds. Treatments of seeds with antagonistic bacterial suspension significantly reduced the infection of *L. theobromae* ($P<0.05$). This study showed that *B. cepacia* has more potential to reduce the fungal infection followed by *B. vietnamiensis* and *B. subtilis*, respectively.

Results in Table-2 showed kenaf seeds germination was increased and infection by *L. theobromae* were reduced in conjunction with antagonistic bacteria treatment. Germination rate of kenaf seeds were increased by the time of inoculation with bacterial isolates. Re-isolation of these 3 bacterial isolates from the soil on NA medium has proved the potential antagonist effect for all test isolates suppress the growth of *L. theobromae*.

Table-2. Mean percentage of seed germination of kenaf seed after treatment with selected diazotrophic bacteria.

Treatments	**Germination rate** (%)			
	3DAI	**9DAI**	**12DAI**	**35DAI**
Inoculated un-germinated seed + *B. cepacia*	55.6[a]	77.8[a]	83.2[a]	83.2[a]
Inoculated un-germinated seed + *B. vietnamiensis*	33.3[ab]	50.0[b]	77.8[a]	77.8[a]
Inoculated un-germinated seed + *B. subtilis*	33.3[ab]	44.4[b]	66.7[a]	66.7[a]
Inoculated seed (control)	16.67[c]	16.67[c]	33.3[b]	33.3[b]

Means followed with same letter are not significantly different at 5% level by Duncan Multiple Range Test

DISCUSSIONS

This study was carried out to observe the potential of diazotrophic bacteria isolated from kenaf rhizospheresand roots in Malaysia. Isolates obtained from kenaf plantation area in Perlis, Malaysia were used as a biological control for *L. theobromae* suppression. Study firmly established that some diazotrophic bacteria which have known as a plant growth promoter, offers benefit to suppress phytopathogens by direct bacterial inoculants introductionas well. Previous studies demonstrated the dual culture assay techniques or analysis on agar plates was the easiest assessment as a preliminary study to determine the antagonist potential of bacteria by evaluation of fungal growth inhibition zone.

Sixty-eight diazotrophic bacteria from kenaf roots were successfully isolated. However, only 3 bacterial strains that displayed the obvious antifungal activity by dual culture assay were selected for further analysis to control black rot disease on kenaf seeds caused by *L. theobromae*. Three selected isolates RFC1a, RKBP3d and IRPP1b showed the fungal inhibition zone within 0.2 cm to 6.0 cm. Other isolates either showed the limit or none antifungal activity towards the dual culture assay (data not shown).

Different inhibitory activity among isolates was influenced by the type of antifungal metabolites production (William and Asher, 1996). Furthermore, the inhibition zone of 3 isolates showed no physical contact between isolates and the pathogen. This was explained that the isolated diazotrophs species could produce certain antifungal metabolites which inhibit the growth of mycelia (Montealegre *et al*., 2003). Moreover, the used of PDA on dual culture assay which enriched with nutrients, proved that no competition might be excluded as a mode of action of the isolates (Lee *et al*., 2008).

Three selected isolates, RKBP3d, RFC1a and IRPP1b were identified as closely related to *Bukholderia cepacia*, *Bukholderia vietnamiensis* and *Bacillus subtilis*, respectively by 16S rDNA sequence amplification. RKBP3d and RFC1a were similar according to morphological observation on NA plate, with yellow colony colour. Figure-5 showed a phenogram of relationship among the isolates and the candidates sequences of various diazotrophic bacteria obtained from NCBI database. RKBP3d and RFC1a were grouped together within the same genera. However, 16S rDNA sequence identification by universal primersboth isolates where genetically different and successfully identified as different species of *Burkholderia*. RKBP3d and RFC1a were identified as *Burkholderia cepacia* and *Burkholderia vietnamiensis*, respectively. Meanwhile IRPP1b was identified as *Bacillus subtilis* and clustered together with other *Bacillus* species obtained from NCBI database.

Several studies were reported the same species of bacteria used as biological control agent.*Bacillus* spp. is one of the endophytic bacteria used to control*Rhizoctonia solani* and *Sclerotium rolfsii* (Pleban *et al*., 1995). Antibiotic and enzyme production by *Bacillus* strains have been reported as the essential element in biological control of phytopathogens. *Bacillus subtilis* possess a lytic factor that lyses the fungal pathogen cell wall (Pukall *et al*., 2005).This fact was supported by Mavingui and Heulin

(1994). The study observed that inhibiting effect of biological agents including *Bacillus* strains on phytopathogens fungi might associated with enzyme production that act against the fungal cell wall.

Antibiotic production was also associated with antagonist characteristics in *Burkholderia* strains. As in *Bacillus* group, *B. cepacia* complex produced antifungal metabolites and siderophores (Parke and Gurian-Sherman, 2001) in order to suppress the growth of fungal pathogen. This species was produced several antibiotics including cepacin (Parker *et al*., 1984), cepaciamide (Jiao *et al*., 1996) and pyrrolnitrin (Raaijmakers *et al*., 2002). *Bukholderia cepacia* has been isolated from various crops. *B. cepacia* isolated from tomato, lettuce, chicory and pumpkin plants rhizosphere have an ability to suppress phytopathogens (Sfalanga *et al*., 1999).

In dual culture assay, antagonistic effect was performed on the basis of interaction of pathogen and antagonist bacteria. Meanwhile, in field condition, pathogen, antagonist bacteria and host plant were resembled together. There was no involvement of host plant in dual culture assay, whereas in the real condition, host plant plays an important role in supporting the introduced antagonists (Anith *et al*., 2003). Hence, the interaction between host plant and antagonist bacterial with the involvement of pathogen in field study will give more realistic view than dual culture assay.

Other study conducted by Quan *et al*. (2006), stated that *B. cepacia* isolated from compost samples was demonstrated to reduce hyphal extension rates of *Rhizoctonia solani*. As well as *B. cepacia*, *B. vietnamiensis* also produces several antibiotics (Miché *et al*., 2000).

For the above reason, all the selected potential antagonist bacteria from dual culture assay were subjected for further evaluation under glasshouse conditions. As in vitro experiment, glasshouse experiment showed varying level of antagonism by bacterial isolates. This condition was showed by increasing the percentage of seed germination. *Bukholderia cepacia* was showed the highest germination of kenaf seed (83%), followed by *B. vietnamiensis* (77%) and *B. subtilis* (66%). Though, all the isolates showed a good antagonistic activity in dual culture, also successful to suppress the disease incidence under in vivo conditions. The antagonist bacteria were able to penetrate the mycelia, colonize and protect the seeds from mycelia penetration.

CONCLUSIONS

In conclusion, we propose that diazotrophic bacteria including *B. cepacia*, *B. vietnamiensis* and *B. subtilis* might be suitable for use as biological control agents against phytopathogenic fungi. Further study should be conducted specifically on mechanism and inhibition factor of antagonist bacteria. Relevant analytical studies would also head to the identification of antibiotic compound or other metabolites produced by the diazotrophs isolates. Further research in glasshouse and field experiment may contribute to the development of biocontrol agent formulation which is suitable for application in tropical condition.

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