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Preliminary screening and characterization of fungi isolated from forest soil as biocontrol agent against *Ganoderma boninense*

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ABSTRACT

Aims: The objective of this study is to investigate the potential of fungi isolated from forest soil as biocontrol against *Ganoderma boninense,* the causal pathogen of basal stem rot disease in *Elaeis guineensis* Jacq. (oil palm).
Methodology and results: Total 195 isolates were isolated from 20 soil samples collected from Crocker Range of Sabah and 54 fungal isolates were identified with 14 of them showed Percentage Inhibition of Radial Growth (PIRG) greater than 50%. A potential fungi (F15) with PIRG of 84.85% was later identified as *Penicillium simplicissimum* using molecular technique. Microscopy examination on *P. simplicissimum* and *G. boninense* interaction showed the evidence on the damage of pathogen hyphae when challenged by *P. simplicissimum*. The secondary metabolites of *P. simplicissimum* which may possibly contribute to this observation were later extracted using hexane, ethyl acetate and acetone and the extracts were tested in agar dilution bioassay (0.2 mg/mL to 1.0 mg/mL) against the pathogen. Ethyl acetate extract gave the highest inhibition to *G. boninense* (14.12 % in 0.4 mg/mL of ethyl acetate extract).
Conclusion, significance and impact of study: This is the first report, on the bioactivity of *P. simplicissimum* isolated from Crocker Range of Sabah against *Ganoderma boninense*, the causal pathogen of basal stem rot disease. Overall, our results indicated that *P. simplicissimum* has the potential to be further investigated as a biocontrol agent against *G. boninense.*

Keywords: Basal stem rot, oil palm, *Ganoderma boninense*, *Penicillium simplicissimum*

INTRODUCTION

Basal stem rot (BSR) is a serious disease prevalent to *Elaeis. guineensis* Jacq. industry that can lead to low productivity and death of plants thus resulting in great economy loss to the producer countries, especially Malaysia and Indonesia. *Ganoderma boninense*, a white rot basidiomycete has been identified as the major causal of BSR in South East Asia. Numerous methods have been attempted to control BSR, but to date, no method gives good control of *Ganoderma* infection in established plantations. Cultural practices such as clean clearing and windrowing are not totally effective in controlling BSR and it only helps to reduce the potential of infection. Chemical control such as trunk injection of systemic fungicides has been reported (Chung, 1990; George *et al.,* 1996) and at most, some systemic fungicides managed to prolong the economic life of BSR palms but failed to give effective control of the disease because treated BSR palms finally were dead. This may be due to the fact that both visibly infected and subclinical palms may already have the disease established by the time treatment is applied and *Ganoderma* has several resting stages such as pseudosclerotia (melanised mycelium) and basidiospores (Susanto *et al*., 2005) that are more resistant to the fungicides. In this paper, we report the bioactivity of a potential biocontrol agent against *G. boninense* isolated from Crocker Range of Sabah. Forest soil has higher microbial population compare to disturbed soil such as plantation soil.

MATERIALS AND METHODS

Soil sampling

Twenty soil samples were collected randomly from Crocker Range forest, Sabah, Malaysia (5.4008° N, 116.1033° E) in the morning. Samples were collected 15 cm in depth from the ground using sterilized pvc pipes (5 cm × 15 cm). Soil samples were packed in ziplock bags

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and stored in a container with ice pack during transportation from sampling site to the University's laboratory.

Isolation of potential antagonists from soil samples

The isolation of microbes of interest was conducted using dilution plate method (DPM). A serial dilution of the soil sampling from 10^{-1} to 10^{-8} was prepared. To prepare a soil dilution with 10^{-1} dilution, 10 g of soil was suspended into 90 mL of sterile distilled water in a 250 mL conical flask. The suspension was then serially diluted to 10^{-8}. An aliquot of 100 uL of each dilution was taken and spread evenly on the surface of potato dextrose agar (PDA) using L-shaped glass spreader. All plates were incubated at room temperature (28±1 °C) for one to two weeks. Colonies formed on the PDA plates were then sub-cultured to obtain a pure culture.

Dual culture assays

The assays were performed on PDA. *G. boninense* mycelial plug (8 mm) was taken from the edge of a seven days old *G. boninense* pure culture which was previously identified by Chong *et al*. (2011) and placed 30 mm from one side of the PDA plate (90 mm). After two days, the potential antagonist fungal mycelial plug (8 mm) of seven days old was placed 30 mm from the *G. boninense* plug. The radial growth of the pathogen in the dual culture was measured after seven days of incubation at room temperature (28±1 °C) and the PIRG was calculated accordingly to Bivi *et al*. (2010). The formula for PIRG is as followed: [(R1- R2)/R1] x 100% where, R1 is the radial growth of *G. boninense* in the control plate while R2 is the radius of the *G. boninense* colony in the direction towards the antagonist colony. Plates with only *G. boninense,* served as controls and each culture assay was conducted in three replicates.

Microscopy examination of the potential biocontrol agent

The interaction of the isolate with highest PIRG against the pathogen was observed using Zeiss EVO® 10MA Scanning Electron Microscopy. The zone of interaction between *G. boninense* and the isolate in dual culture assay (day seven) was prepared for observation. *G. boninense* fungal mycelia in the middle of the agar plate were cut to approximately 1.0 cm × 1.0 cm in size using scalpel. The samples were then air dried in laminar flow at room temperature (28±1 °C) for about three to four hours. The samples were mounted onto aluminium stubs using conductive double-sided adhesive carbon tabs (NISSHIN EM. CO. LTD). The stubs were then sputter coated with gold-palladium (Emitech K550x carbon coater) from different angles to ensure a complete and uniform film over sample surfaces. Coated surfaces of samples were viewed at 15 kV voltage.

Identification of biocontrol agent

Fungi (F15) with the highest PIRG was identified using molecular technique. The method for DNA extraction and PCR amplification of ITS was following the one described by Latifah *et al*. (2002) with some modifications. Primer used were ITS 1: 5'- TCC GTA GGT GAA CCT GCG G - 3' (Gardes and Bruns, 1993) and ITS 4: 5'- TCC TCC GCT TAT TGA TAT GC -3' (Singh, 2001). The PCR thermal cycling condition was started with initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 40 s, and extension at 72 °C for 50 sec. The final extension was at 72 °C for 10 min. The PCR product was sent to First BASE laboratories Sdn Bhd in Selangor, Malaysia for DNA sequencing. Forward and reverse sequences were assembled in BioEdit v.7.2.5.0 and used for BLASTN with NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Sequences found in the database with the highest nucleotide similarities (96% or more) were considered as the closest relative of the query sequence.

Antimicrobial assay of crude extracts

Fungi with the highest PIRG was cultured in potato dextrose broth for seven days. The broth culture was then extracted using hexane, ethyl acetate and acetone respectively. The solvent phase (hexane and ethyl acetate) were separated from water phase (medium) using separatory funnel. Solvent phase (hexane and ethyl acetate) were collected and dried using rotary evaporator (Stuart®) at 37 °C while the culture filtrate with acetone was collected and dried in freeze dryer (Thermo ModulyoD). The sample was pre-freezed at –20 °C before dried in freeze dryer. Dried crude extracts were weighed and used for bioassays. Agar dilution method (0.20 mg/mL to 1.0 mg/mL) was used to test the antimicrobial efficacy of the extracts (dissolved in DMSO) on *G. boninense.* The percentage of inhibition growth expressed was calculated based on the following formula, [Mean diameter of *G. boninense* in control plate - Mean diameter of *G. boninense* in treatment plate] / Mean diameter of *G. boninense* in control plate x 100 (Bivi *et al.*, 2010).

RESULTS

Dual culture assay of the potential antagonist against *G. boninense*

Out of the total of 195 isolates from the 20 soil samples with different morphology, there were 54 fungal isolates (Data not shown). However, only 14 fungal isolates showed PIRG greater than 50% against *G. boninense*. Fungal isolate with highest PIRG was given an identity code as F15 (PIRG=84.85%). The dual culture assay of F15 against *G. boninense* is illustrated in Figure 1.

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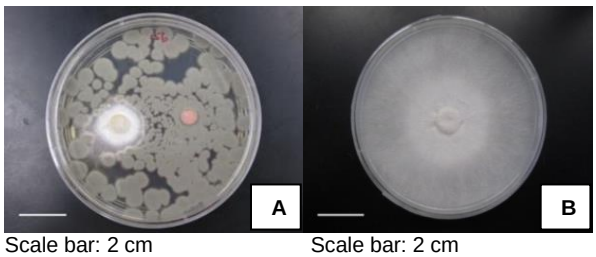

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Figure 1: A: Fungi isolate, F15 with the highest PIRG (84.85%), B: Control plate of *G. boninense.*

Morphological changes of *G. boninense* during the interaction with F15

Interaction zone of antagonist (F15) and *G. boninense* in dual culture assays were examined under scanning electron microscope. Figure 2a is the control *G. boninense* with dense fungal mat. Figure 2b is the mycelial of F15 and *G. boninense* at the interaction zone. Arrows were showing the conidia of F15 (Figure 2b) and circled area in Figure 2c was the damaged hyphae of *G. boninense*.

Identification of F15 antagonist with the highest PIRG to *G. boninense*

The gel electrophoresis images from crude DNA of F15 is shown in Figure 3a. The gel electrophoresis image from PCR amplification of F15 using ITS 1 and ITS 4 primer was shown in Figure 3b. The F15 amplicon was in between 500 bp to 750 bp. Forward and reverse sequences were assembled in BIOEDIT v.7.2.5.0. Figure 4 shows the assembled sequences of F15 that was used for the BLASTN in NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Sequences found in the database with the highest nucleotide similarities were considered as the closest relative of the query sequence. The identity of F15 was confirmed using molecular technique as *Penicillium simplicissimum* (Table 1).

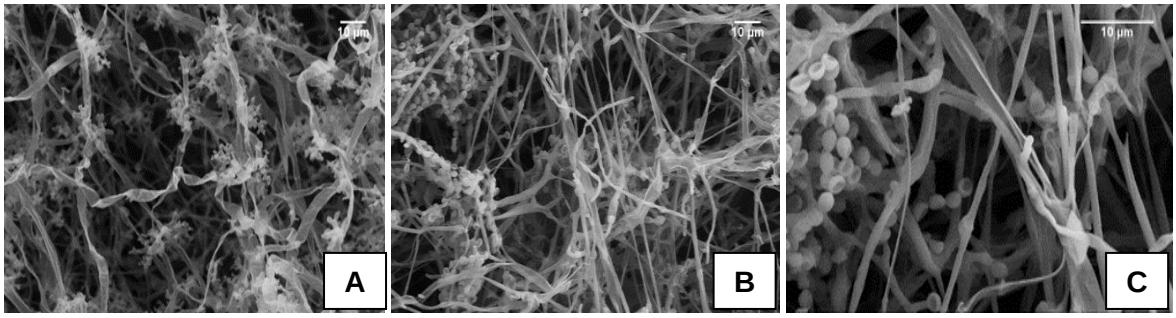

Figure 2: Micrograph of *G. boninense* under scanning electron microscopy, A: Morphology of *G. boninense* hyphae without interact with the antagonist (Control) at 2,000x magnification; B: Morphology of *G. boninense* hyphae interact with F15 at 2,000×magnification; C: Morphology of *G. boninense* hyphae interact with F15 at 5,000× magnification

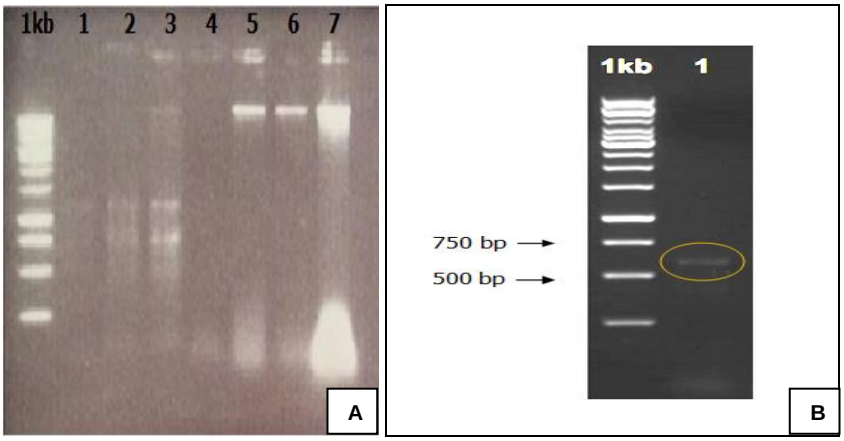

Figure 3: Gel image of F15 genomic DNA and PCR product; A, Lane 6 represents genomic DNAs; B, PCR amplification using the pairs of primers ITS 1/ITS 4. PCR product of F15 is circled.

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TATCAGACGTGCTACTGCATGCAGAGGTCACCATGTGAAGATTGATTGGGGTCTCCGGCGGGCGCCGGCCGGGCCTACTGA

GCGGGCGACAAACCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCCCTGCCTTTCCGGCCCGCCACCCGGGAGCCGG

GGGGCGCAAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCACGGAATACCAGGAG

GCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGA

TGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTTATTTAATCTGCTCAGACTGCAATCTTCAGACAGAGTTCAAT

GGTGTCTTCGGCGGGCGCGGGCCCGGGGGCAGGTGCCCCCCGGCGGCCGTGAGGCGGGCCCGCCGAATCAACAAGGTA

TAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACCCTGTAATGATCCTTCCCCAAGTTCCCCTATCGAAGACCAT

TTACTAAACTACAGAGCTCACGCAACGCCC

Figure 4: F15 sequences used for BLAST search at NCBI GenBank database.

Table 1: Top five identity of F15 from NCBI Genbank database.

Isolate	Accession No.	Description	Max score	Total score	Query value	E value	Ident
F15	HQ607998.1	*Penicillium simplicissimum* isolate CY160 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	863	863	89%	0.0	96%
	HQ392489.1	*Penicillium simplicissimum* strain LP42 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region.	854	854	87%	0.0	96%
	GU594654.1	*Penicillium* sp. FA 21 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	854	854	89%	0.0	96%
	KC156903.1	*Penicillium* sp. RPS29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	852	852	89%	0.0	96%
	HQ392487.1	*Penicillium simplicissimum* strain LP33 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region.	850	850	87%	0.0	96%

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Antimicrobial activity of crude extract of *P. simplicissimum* F15 against *G. boninense*

Penicillium simplicissimum F15, ethyl acetate, and hexane extracts showed the highest inhibition with no significance differences (p > 0.05) among the solvents (Figures 5 and 6). The lowest growth inhibition of *P. simplicissimum* F15 extracts to *G. boninense* was 0.00 % from acetone extract while the highest growth inhibition was 14.12 % from ethyl acetate extract.

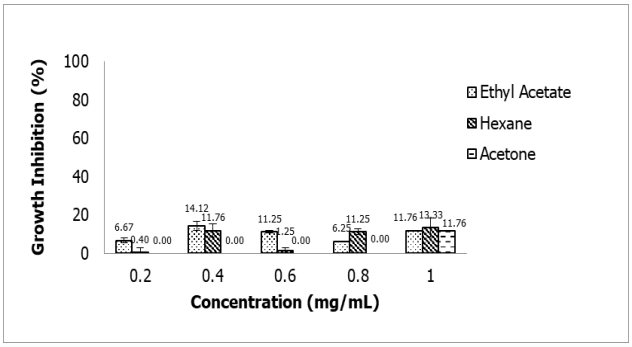

Figure 5: Percentage Inhibition of *G. boninense* growth at different concentrations of *P. simplicissimum* solvent extracts.
Note: Control *G. boninense* (without DMSO) is not significantly different to control *G. boninense* (with DMSO) according to Tukey HSD posthoc test.

DISCUSSION

Malaysia's land areas are made up of 62 % forest and this includes some of the world's oldest rainforests. The Crocker Range is a Sabah national park with approximately 139,919 hectares (Thiessen, 2012) with densely forested terrain, mainly lowland, hill and montane forests, is a forest reserved. Forest soils contain thousands of species of microorganisms (Staddon *et al*., 1996). Most terrestrial ecosystem functions occur in the soil, which possesses the greatest amount of biodiversity on earth. Soil microorganisms represent a considerable fraction of the living biomass on earth, with 10^3–10^4 kg of microbial biomass per hectare of surface soils (Ferreira *et al*., 2007). Forest soil has higher microbial population compare to disturbed soil such as plantation soil. The relatively dense growth of plants and higher accumulation of litter on the forest floor and distribution of fine roots in undisturbed forest favor the growth of microorganisms (Dkhar *et al*., 2012). In the soil ecosystem, bacterial and fungal communities differ in their spatial distributions with fungal taxa more distinctly confined to either the litter or the organic horizon of soil and more heterogeneously distributed in the ecosystem (Petr *et al*., 2012).

The slow growth of *G. boninense* gives *P. simplicissimum* F15 an advantage against *G. boninense* in the competition for space and nutrients. The conidia of F15 overgrow the mycelium of *G. boninense* (Figure 2b), however, there was no lysis and destruction of cell wall of *G. boninense* was observed. Only several damaged hyphae observed as shown in Figure 2c and there were no antler-like hyphae observed as compared to control *G. boninense* (Figure 2a).

From NCBI nucleotide blast, it was suggested that F15 was very likely to be *P. simplicissimum* due to the higher identical percentage, higher total score, higher maximum score and lower E-value compare to other possible identities as shown in Table 1. Molecular (DNA sequence) data has emerged as crucial information for the taxonomic identification of microbes, with the nuclear ribosomal internal transcribed spacer (ITS) region being the most popular marker for fungal identification (Nilsson *et al*., 2014). Molecular identification of fungi usually relies, at least in the first attempt, on sequencing the nuclear ribosomal ITS region, the formal fungal barcode (Schoch *et al*., 2012). Among the regions of the ribosomal cistron, the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter and intraspecific variation (Schoch *et al*., 2012). This study is targeted on the amplification of ITS region of rRNA gene of selected antagonistic fungal isolate using ITS1 and ITS4 universal primers (White *et al*., 1990). Amplified products of size in the range of 500 to 750 bp were produced by the primers. ITS sequences shared among different species have already been documented in species rich *Pezizomycotina* genera with shorter amplicons, such as the economically important genera *Cladosporium* (Schubert *et al*., 2007), *Penicillium* (Skouboe *et al*., 1999), and *Fusarium* (O' Donnell and Cigelnik, 1997).

In order to understand the bioactive compounds from the isolate (culture in submerge fermentation), solvent extraction was performed. To extract wide range of molecules from the isolate, three different solvents were used to extract broader range of organic molecules based on their polarities. From the current study, ethyl acetate extract of *P. simplicissimum* F15 gave the highest inhibition against *G. boninense*, suggesting that the inhibitory compounds from these isolates are better extracted with ethyl acetate. Statistically, hexane extract of *P. simplicissimum* F15 showed no significant difference with ethyl acetate extract. The used of organic solvents is reported to be more efficacious in extraction of antimicrobial compounds when compared to water extracts (Parekh *et al*., 2005). To solubilize the hydrophilic compounds, polar solvents, such as methanol, ethanol or ethyl acetate are required. Hence, the organic solvent ethyl acetate was thought to enhance the solubility of the compound. The ethyl acetate extract of *P. simplicissimum* F15 showed effective results against the pathogen and this could be related to the presence of bioactive metabolites present in *P. simplicissimum* F15 are soluble in ethyl acetate and might be a moderately polar to polar in nature (Bibi *et al*., 2011). This also indicates that ethyl acetate extracts have higher concentration of active components and thus have greater potency against the pathogen.

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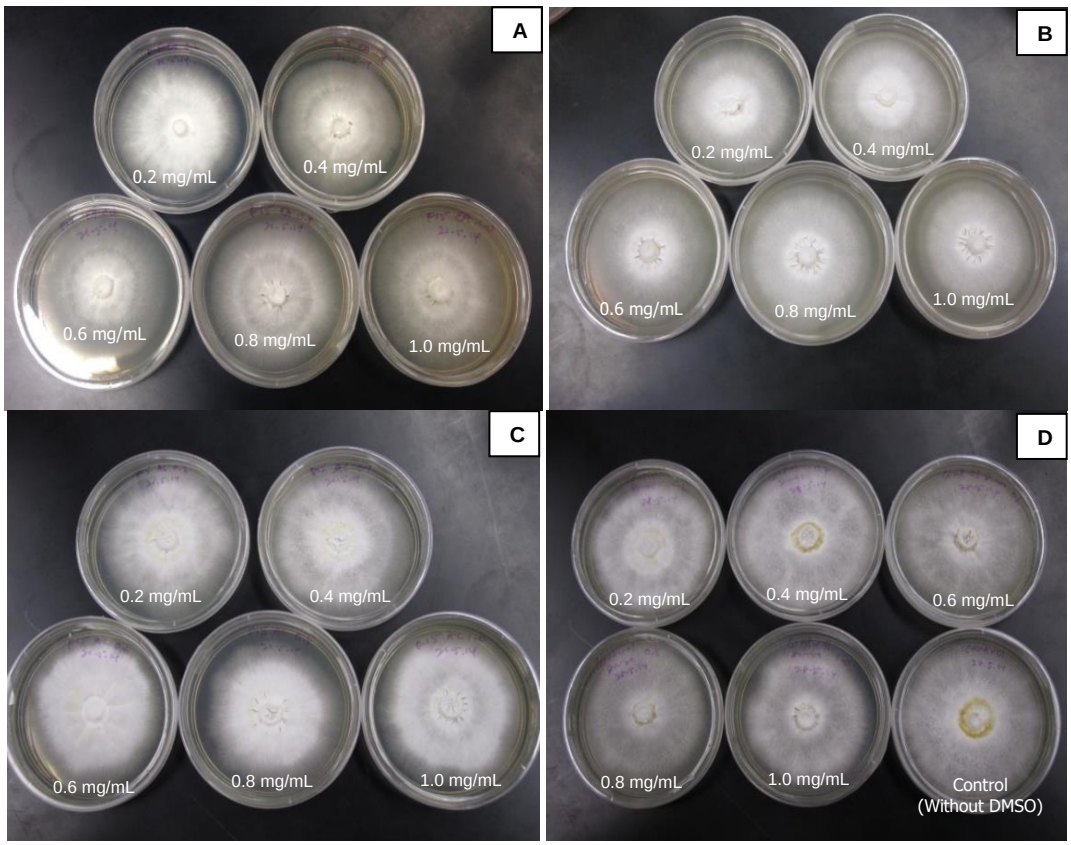

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Figure 6: Antimicrobial assay (*P. simplicissimum* F15) of different solvents extract at five different concentrations against *G. boninense*. A: Ethyl acetate extract; B: Hexane extract; C: Acetone extract; D: Control. DMSO denotes Dimethyl Sulfoxide. All extracts were resuspended in 99.8% of DMSO.

Even though PIRG from dual culture assay of *P. simplicissimum* F15 was 84.85 %, the crude extract antimicrobial activity assay showed poor inhibition against the pathogen. Therefore, the previous PIRG of *G. boninense* caused by *P. simplicissimum* F15 may more likely due to the competition between *P. simplicissimum* F15 and *G. boninense* for space and nutrient in the agar plate. In addition to this, there was no destruction of fungal cell wall or lysis observed under scanning electron microscope as shown in Figure 2. *Penicillium simplicissimum* F15 might not secrete remarkable metabolites against *G. boninense* during this interaction. However, perhaps the reason that the extracts were not active was due to low concentration of the active compounds or the secretion may be too low for detection of activity (Molinari, 2009). The presence of other compounds could also inhibit the test and the presence of polar functional groups in the metabolites can cause them instantly soluble in water than other less polar organic solvents (Gurung *et al*., 2010). Besides that, bioactive compounds released by *P. simplicissimum* F15 might not effective against *G. boninense*. For example, paraherquamides is one of the secondary metabolites produced by *P. simplicissimum* which containing the bicyclo [2.2.2] diazaoctane core that normally contribute to insecticidal, antitumor, anthelmintic, calmodulin inhibitory and antibacterial activities (Lin *et al*., 2008).

There are many other factors that will affect secondary metabolites production. For example, types of media, incubation time, size of inocula and culturing method. The production of secondary metabolites by fungi, in general, is often affected by various growth conditional factors mainly the fermentation medium (Jonathan and Fasidi, 2003; Vahidi *et al*., 2004). The growth and secondary metabolites production of *Aspergillus terreus*, *Penicillium janthinellum* and *Penicillium duclauxii* were significantly affected by the type of the growth medium (Zain *et al*., 2009). Growth medium (potato dextrose broth) used in this study might not favourable for antimicrobials production from *P. simplicissimum.*

CONCLUSION

Fungi isolated from Crocker Range forest soil with the highest PIRG (84.85%) was identified as *P. simplicissimum*. *G. boninense* might be inhibited by competition with *P. simplicissimum* F15 for space and nutrients as the conidia of *P. simplicissimum* F15

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overgrow the mycelium of *G. boninense* and there were no lysis or destruction of *G. boninense* cell wall observed. Ethyl acetate extract of *P. simplicissimum* F15 gave the highest inhibition against *G. boninense*, suggesting the inhibitory compounds from *P. simplicissimum* are better extracted with polar solvent. The potential of *P. simplicissimum* can be further explored and bioactive compounds can be further purified, identified and characterised for future study.

REFERENCES

Bibi, Y. Nisa., S., Chaudhary, F. M. and Zia, M. (2011). Antibacterial activity of some selected medicinal plants of Pakistan. *Biomedical Central Complementary and Alternative Medicine* **11(52), 1-7.**

Bivi, M. R., Farhana, M. S. N., Khairulmazmi, A. and Idris, A. S. (2010). Control of *Ganoderma Boninense*: A causal agent of basal stem bot disease in oil palm with endophyte bacteria *in vitro*. *International Journal of Agriculture and Biology* **12, 833-839**.

Chong, K. P., Lum, M. S., Foong, C. P., Wong, C. M. V. L., Atong, M. and Rossall, S. (2011). First identification of *Ganoderma boninense* isolated from Sabah based on pcr and sequence homology. *African Journal of Biotechnology* **10(66), 14718-14723.**

Chung, G. F. (1990). Preliminary results on trunk injection of fungicides against *Ganoderma* basal stem rot in oil palm. *In: Proceedings of Ganoderma Workshop, Kuala Lumpur*. **pp. 81 -97.**

Dkhar, M., Dkhar, M. S. and Tynsong, H. (2012). Microbial activities and nutrient dynamics in sacred forest of Meghalaya. *Indian Journal of Innovations and Development* **1(3), 175-185.**

Ferreira, R. B., Monteiro, S., Freitas, R., Santos, C. N., Chen, Z., Batista, L. M., Duarte, J., Borges, A. and Teixeira, A. R. (2007). The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* **8(5), 677-700.**

Gardes, M. and Bruns, T. (1993). ITS Primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2(2), 113-118.**

George, S. T., Chung, G. F. and Zakaria, K. (1996). Updated results (1990–1995) on trunk injection of fungicides for the control of *Ganoderma* basal stem rot. In: PORIM International Palm Oil Congress (Agriculture). Palm Oil Research Institute of Malaysia, Bangi. **pp. 5 08-515.**

Gurung, T. D., Sherpa, C., Agrawal, V. P. and Lekhak, B. (2010). Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar, Mount Everest region. *Nepal Journal of Science and Technology* **10, 173-182.**

Jonathan, S. G. and Fasidi, I. O. (2003). Effect of carbon, nitrogen and mineral sources on growth of *Psathyerella atroumbonata*. *African Journal of Biomedical Research*. **6, 85-90.**

Latifah, Z., Harikrishna, K., Tan, S. G, Abdullah, F. and Ho, Y. W. (2002). Restriction analysis and sequencing of the ITS Regions and 5.8S Gene of rDNA of *Ganoderma* isolates from infected oil palm and coconut stumps in Malaysia. *Annals of Applied Biology* **141, 133-142.**

Lin, Z. J., Wen, J. N., Zhu, T. J., Fang, Y. C., Gu, Q. Q. and Zhu, W. M. (2008). Chrysogenamide a from an endophytic fungus associated with cistanche deserticola and its neuroprotective effect on SH-SY5Y Cells. *The Journal of Antibiotics* **61, 81-85.**

Molinari, G. (2009). Natural products in drug discovery: Present status and perspectives. *Advances in Experimental Medicine and Biology* **655, 13-27.**

Nilsson, R. H., Hyde, K. D., Pawłowska, J., Ryberg, M., Tedersoo, L., Aas, A. B., … and Abarenkov, K. (2014). Improving ITS sequence data for identification of plant pathogenic fungi. *Fungal Diversity* **67(1), 11-19.**

O' Donnell, K. and Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7, 103-116.**

Parekh, J., Jadeja, D. and Chanda, S. (2005). Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. *Turkish Journal of Biology* **29, 203-210.**

Petr, B., Miroslav, K., Martina, S., Jan, K., Vendula, V., Tomas, V., Lucia, Z., Jaroslav, S., Jakub, R., Cestmir, V. and Jana, V. (2012). Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. *International Society for Microbial Ecology Journal*. **6(2), 248-258.**

Schoch, C. L., Seifert, K. A., Huhndorf, S. Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. and Consortium, F. B. (2012). Nuclear ribosomal Internal Transcribed Spacer (ITS) region as a universal DNA barcode marker for fungi. *In: Proceedings of the National Academy of Sciences*. **109, 6241-6246.**

Schubert, K., Groenewald, J. Z., Braun, U., Dijksterhuis, J., Starink, M., Hill, C. F., Zalar, P., Hoog, G. S. and Crous, P. W. (2007). Biodiversity in the *Cladosporium herbarum* complex (Davidiellaceae, Capnodiales), with standardisation of methods for *Cladosporium* taxonomy and diagnostics. *Studies in Mycology* **58, 105-156.**

Singh, V. K. and Kumar, A. (2001). PCR primer design. *Molecular Biology* **2, 27-32.**

Skouboe, P., Frisvad, J. C., Taylor, J. W., Lauritsen, D., Boysen, M. and Rossen, L. (1999). Phylogenetic analysis of nucleotide sequences from the ITS Region of *Terverticillate Penicillium* species. *Mycological Research* **103, 873-881.**

Staddon, W. J., Duchesne, L. C. and Trevors, J. T. (1996). Conservation of forest soil microbial diversity: the impact of fire and research needs environmental review **4, 267-75.**

Susanto, A., Sudharto, P. S. and Purba, R. Y. (2005). Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. *Mycopathologia* **159(1), 153-157.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Thiessen, T. (2012). Borneo: Sabah-Brunei-Sarawak. USA: The Globe Pequot Press Inc. **pp. 67.**

Vahidi, H., Kobarfard, F. and Namjoyan, F. (2004). Effect of cultivation conditions on growth and antifungal activity of *Mycena leptocephala*. *African Journal of Biotechnology* **3, 608-609.**

White, T. J, Bruns, T., Lee, S. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* PCR Protocols: A Guide to Methods and Applications. Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (eds.). Academic Press, Inc, New York. **pp. 315-322.**

Zain, M. E., Razak, A. A., El-Sheikh, H.H., Soliman, H. G. and Khalil, A. M. (2009). Influence of growth medium on diagnostic characters of *Aspergillus* and *Penicillium* species. *African Journal of Microbiology Research* **3(5), 280-286.**