



Identification and characterization of antifungal compounds from a *Burkholderia* strain against plant pathogenic fungi

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ABSTRACT

Aims: Biocontrol of fungal plant pathogens using beneficial microorganisms is a safer alternative over synthetic fungicides. PHP12 is a bacterial strain isolated from healthy oil palm rhizosphere and is closely related to the recently described *Burkholderia stagnalis*, a member of the *Burkholderia cepacia* complex. This study aimed to characterize the antifungal activity spectrum of PHP12 and identify the antifungal compounds produced by the strain.

Methodology and results: The antifungal activity of PHP12 was characterized by growing fungal strains in the presence and absence of PHP12 and measuring the radius of the antifungal zone. PHP12 inhibited the growth of fungal pathogens including *Ganoderma boninense*, *Curvularia oryzae*, *Phellinus noxius* and *Colletotrichum capsici*. However, PHP12 did not inhibit the growth of *Trichoderma asperellum*, a known fungal biocontrol agent. The antifungal compounds of PHP12 were precipitated using ammonium sulfate and further purified with HPLC followed by identification using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS). The LC/ESI-MS analysis showed the presence of an oligopeptide with a molecular weight of 1210.63 Da. The peptide consists of heavily modified amino acids that are linked by a hexose residue.

Conclusion, significance and impact of study: Although characteristics of the antifungal compounds are similar to other antifungal peptides from *Burkholderia* such as occidiofungin, there have been no reports of antifungal peptides from *B. stagnalis* with the corresponding molecular weight or fragmentation profile. The novelty of the compound, as well as its antifungal spectrum, makes PHP12 an interesting strain to be investigated further as a biocontrol agent.

Keywords: Antifungal compounds, biocontrol, *Burkholderia stagnalis*, mass spectrometry, plant pathogen

INTRODUCTION

With more than 8,000 species of fungi known to cause plant diseases, fungal diseases greatly impact overall plant production and therefore reduction of food sources and financial income. Several plant pathogenic fungi such as *Ganoderma boninense*, *Fusarium oxysporum*, and *Phellinus noxius* are pathogens of oil palms that can kill infected trees when the palms are halfway through their economic life (Aderungboye, 1977). *Curvularia* sp. is a pathogenic fungal species that causes brown leaf spots in rice (Majeed *et al.*, 2015) and causes leaf spot disease in oil palm seedlings (Omamor *et al.*, 2007). Other fungal diseases are Sclerotium rot in melons that is caused by *Sclerotium rolfsii* (Darma *et al.*, 2016), root rot in herbaceous crops caused by *Pythium* sp. (Agris, 2005)

and anthracnose in chili plants caused by *Colletotrichum* sp. (Than *et al.*, 2008).

There has been a growing concern in public regarding the use of chemical pesticides to control these fungal pathogens. The use of synthetic fungicides such as hexaconazole creates major problems such as the emergence of fungicide-resistant fungi, accumulation of carcinogenic residues in commercial products and damage to beneficial soil microorganisms (Maznah *et al.*, 2015). A potentially harmless alternative is the use of biocontrol agents in the form of antagonistic microorganisms to inhibit fungal pathogens. Several fungal strains of *Trichoderma* have been reported to be able to inhibit fungal pathogens through antibiosis, parasitism, and competition for nutrients (Ghisalberti and Rowland, 2004). Several *Bacillus* species produce

lipopeptides that act as a surfactant that disrupt the membrane of pathogenic fungi (Romero *et al.*, 2007). The species *Burkholderia cepacia* has been considered as a biocontrol agent against *Rhizoctonia* by producing the antifungal pyrrolnitrin (Hwang *et al.*, 2002).

In this study, we characterized a soil bacterium that is designated as isolate PHP12 that was isolated from soil in an oil palm plantation that was free from *Ganoderma* infestation. Soil bacteria belonging to the genera *Burkholderia*, *Bacillus* and *Azotobacter* can act as plant-growth promoters and are also ideal candidates for biocontrol (Glick and Bashan, 1997). It could be due to their living environment in which they have to compete with fungi to colonize roots. In this paper, we discuss in more detail the identification of the isolate PHP12, its antifungal activity, and the elucidation of antifungal compounds produced by PHP12.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and reagents were of analytical grade or the highest grade available. Ammonium sulfate and acetonitrile were purchased from Merck (Darmstadt, Germany). Trifluoroacetate (TFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Difco™ Potato Dextrose Agar (PDA) was purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Analytical-grade water was obtained from ultrafiltration using Milli-Q® Integral Water Purification System for Ultrapure Water (Millipore; Molsheim, France).

Isolation and identification of PHP12

Isolation of PHP12 was performed based on the protocol described by Cordova-Kreylos *et al.* (2013). The soil sample was collected from Permata Hijau Plantation (PHP), West Sumatra, Indonesia, from a healthy oil palm rhizosphere 30 cm below the soil surface. The PHP12 isolate was maintained on King's B agar at 30 °C or in Luria broth (LB) medium with 20% (v/v) glycerol at -80 °C for long term preservation.

The PHP12 isolate was identified by sequencing the 16S rRNA gene using the 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1387R (3'-GAGTTTGATCCTGGCTCAG-5') primers (Macrogen, Korea). The gene sequencing was carried out using the ABI 3130 Genetic Analyzer (Thermo Scientific). Identification of strain PHP12 was conducted using nucleotide Basic Local Alignment Search Tool (BLASTn; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). To determine the evolutionary relationship of PHP12 within the *Burkholderia*, 16S rRNA sequences from 23 different *Burkholderia* species were retrieved from National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>). 16S rRNA sequence of *Ralstonia solanacearum* was used as an outgroup. Sequences were aligned using ClustalW in Mega X software (Kumar *et al.*, 2018). The phylogenetic tree was

constructed using the Neighbor-Joining method. Tree topology was inferred with the Tamura-Nei correction model (Tamura and Nei, 1993) with 1,000 bootstrap replications based on the best substitution model.

In vitro antifungal assay of PHP12 against several species of fungi

A single colony of PHP12 was streaked over King's B agar and incubated for 48 h at 30 °C. Single colonies were then transferred to 10 mL of King's B liquid media and incubated overnight. After incubation period was complete, PHP12 culture was adjusted to OD 0.5 at 600 nm, and 100 µL of culture was inoculated into 50 mL King's B broth followed by 24 h incubation at 30 °C.

Antifungal activity of PHP12 was tested using the agar well diffusion assay that is modified from the dual culture assay described by Katoch and Pull (2017). The overnight PHP12 culture was centrifuged at 10,000× *g* for 2 min, and the supernatant was decanted and boiled for 10 min to deactivate the remaining microorganisms. The boiled supernatant (100 µL) was dispensed into a pipette-tipped well in PDA plate that was already inoculated with a mycelial plug of actively growing tested fungus at the center of the medium. Eleven fungal species from the culture collection of PT Wilmar Benih Indonesia (*G. boninense* B29, *Curvularia oryzae*, *P. noxius*, *Colletotrichum capsici*, *Neurospora crassa*, *Pythium acanthicum*, *Trichoderma asperellum*, *S. rolfii*, *Rhizopus oligosporus*, *Fusarium oxysporum* and *Saccharomyces cerevisiae*) were used in this experiment. Fungal cultures were incubated at 28 ± 2 °C for 6 ± 2 days. After the incubation period, inhibition zones (mm) were measured. Antifungal testing against *S. cerevisiae* was done as follows: a single colony of *S. cerevisiae* was suspended in 1 mL of 0.85% (w/v) sterile saline water. Yeast suspension (100 µL) was spread on PDA media. PDA was bored using the same methods as previously described, and PHP12 cell-free supernatant (100 µL) was added to the well. As a positive control, 100 µL of commercial fungicide Dithane™ Rainshield™ (containing mancozeb 75% (w/w); Dow AgroSciences, Australia) was added into the PDA wells. King's B liquid medium was used as a negative control. The antifungal activity of the positive control was also tested against *S. cerevisiae*. The diameter of the growth inhibition zone was measured after incubation at 30 °C for 48 h. For each antifungal test, e.g. PHP12 cell-free supernatant tested against *G. boninense*, two wells were used in each plate and the plate was duplicated. Thus, for each antifungal test, four wells were used. The mean for each fungal inhibition zone was therefore derived from average of four inhibition zones.

Extraction of antifungal compounds

The overnight PHP12 culture was centrifuged at 10,000× *g* for 10 min to collect cell-free culture supernatant. Precipitation of the antifungal compounds was carried out by adding ammonium sulfate into the cell-free culture to the concentration of 20% (w/v). The mixture was stirred

on ice for one hour, followed by centrifugation at 10,000× g for 15 min. The pellet was collected, and ammonium sulfate was added into the supernatant to the concentration of 40% (w/v). After stirring and centrifugation, the pellet was collected again, and the supernatant was subjected to another two steps of precipitation with ammonium sulfate at 60% and 80% (w/v). Pellets were collected from the four fractions, each resuspended in 35% (v/v) acetonitrile and concentrated to 20 times of the original volume. The suspensions were tested for antifungal activity against *S. cerevisiae*.

Reversed-phase-high performance liquid chromatography (RP-HPLC)

The four fractions obtained from ammonium sulfate precipitation were subjected to separation by RP-HPLC. The suspension was filtered with a syringe using a 0.2 µm nylon filter (Millex® Syringe Filters; Millipore). The filtrate was injected into analytical HPLC with ZORBAX 300SB C-18 column, 5 µm, 4.6 × 150 mm (Agilent; Santa Clara, CA, USA) at 30 °C connected to Agilent 1200 Series (Agilent) and diode array detector (DAD) and detected at the wavelength of 220 nm. HPLC analysis was carried out with a gradient of 0.1% (v/v) TFA and acetonitrile (9:1) with a flow rate of 0.5 mL/min. The gradient was as follows: 10% acetonitrile for 1.6× column volume, 10-90% acetonitrile for 5.4× column volume, 10% acetonitrile for 2× column volume. The total run time was 45 min. Sub-fractions were collected using a fraction collector. The mobile phase of the collected fractions was evaporated using a vacuum evaporator, and the concentrated extract was resuspended in 35% (v/v) acetonitrile. The collected sub-fractions were tested for antifungal activity against *S. cerevisiae*.

Liquid chromatography – electrospray ionization/mass spectrometry (LC-ESI/MS) analysis

The sub-fractions were analyzed using an Ultimate 3000 UPLC (Thermo Scientific) coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) equipped with an ESI source. Samples were diluted in acetonitrile 30% to the concentration of 100 ppm. The separation was performed on an analytical Acclaim PepMap300 C18 column, 3 µm, 1 × 150 mm. Mobile phase A was 0.1% formic acid in the water, and mobile phase B was 0.1% formic acid in acetonitrile. The ratio of A and B used for the gradient was: 98:2 (A:B) to 60:40 over 2 min; to 30:70 over 20 min; to 1:99 over 1 min, and held at 1:99 for 2 min before re-equilibrating with 98:2 for 4 min. The LC flow rate was 50 µL/min, the column temperature was 45 °C, and the sample injection volume was 5 µL. The LC-MS full scan was performed in positive mode from *m/z* 100 to 1500 with sheath gas at 60 and aux gas at 10 (arbitrary units), spray voltage at 3.3 kV and capillary temperature at 275 °C. The normalized collision energy was set at 25.

RESULTS

PHP12 is a member of the *B. cepacia* complex

Identification of PHP12 was conducted based on 16S rRNA. The sequence of 16S rRNA showed that PHP12 is most closely related to *B. stagnalis* with 99% similarity (Figure 1). The strain is also closely related to other *Burkholderia* species, namely *B. pyrrocinia*, *B. stabilis*, *B. vietnamensis*, and *B. cepacia*. Based on the result, PHP12 can be considered as a member of *B. cepacia* complex (Bcc), a group of Gram-negative bacteria that consist of species closely related to *B. cepacia*.

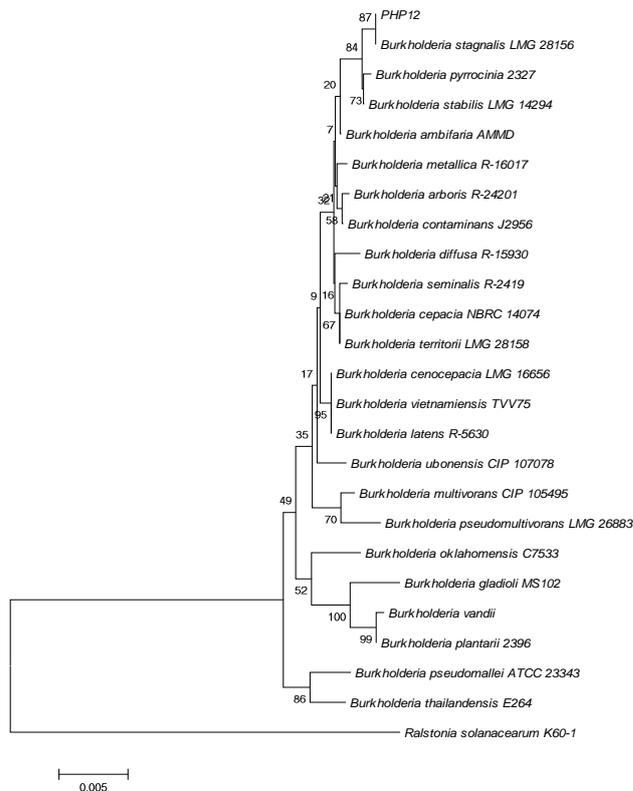


Figure 1: Phylogenetic tree showing the position of PHP12 relative to other *Burkholderia* species. 16S rRNA sequences from 23 different *Burkholderia* species were retrieved from National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>). Sequences were aligned using ClustalW in Mega X software (Kumar *et al.*, 2018). The phylogenetic tree was constructed using the Neighbor-Joining method. Tree topology was inferred with the Tamura-Nei correction model (Tamura and Nei, 1993) with 1,000 bootstrap replications based on the best substitution model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

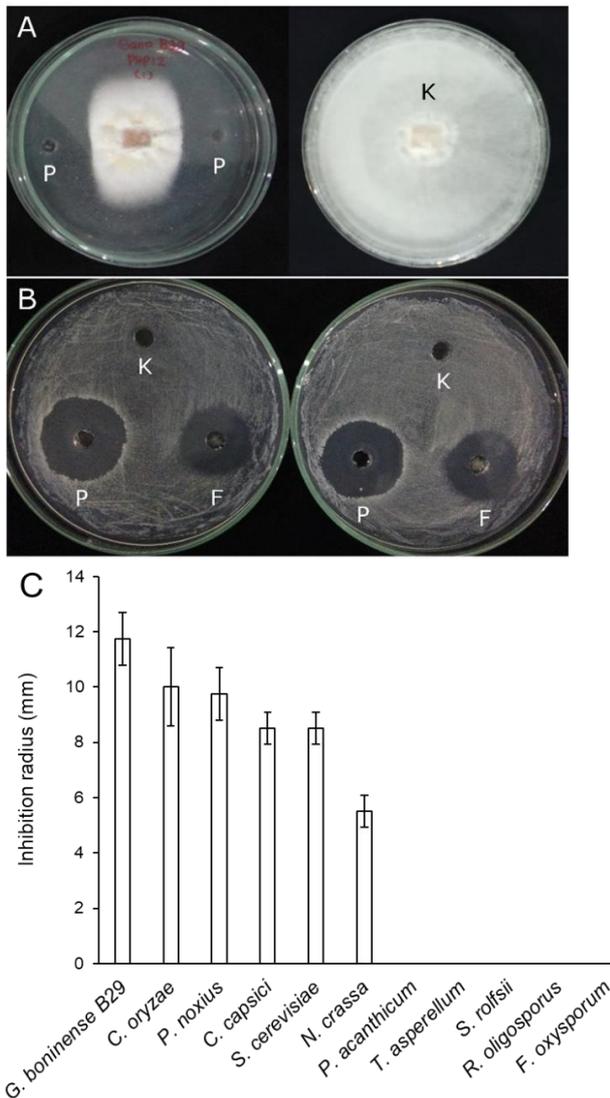


Figure 2: Antifungal assay of PHP12 cell-free culture supernatant against fungi. (A) *G. boninense* B29 grown on PDA incubated at 28 °C for 6 ± 2 days in the presence (left) and absence (right) of PHP12 cell-free culture supernatant. (B) Antifungal activity of PHP12 cell-free culture supernatant, positive control Mancozeb 0.2 g/L (diluted Dithane® M45) and King's B medium as a negative control against *S. cerevisiae* grown on PDA at 30 °C for 48 h. K = negative control; P = PHP12; F = commercial Mancozeb fungicide (positive control). (C) The inhibition zone of PHP12 cell-free culture supernatant against various fungal species. Error bars are standard errors of the means (n=4).

The spectrum of antifungal activity of PHP12

Boiled PHP12 culture was tested against 11 fungal species to determine the antifungal activity of PHP12. The tested fungal species consisted of model organisms (*S.*

cerevisiae, *R. oligosporus* and *N. crassa*), biocontrol agent (*T. asperellum*) and plant pathogenic fungi (*G. boninense* B29, *C. oryzae*, *P. noxius*, *C. capsici*, *P. acanthicum*, *S. rolfisii* and *F. oxysporum*). Cell-free supernatant of PHP12 inhibited the growth of *G. boninense* B29, while there was no inhibition shown by the negative control (King's B medium) (Figure 2A). Cell-free supernatant of PHP12 also inhibited the growth of *S. cerevisiae* as did also a commercial fungicide Mancozeb 0.2 g/L (diluted Dithane® M45) as a positive control (Figure 2B). Cell-free supernatant of PHP12 inhibited six fungal species namely *G. boninense*, *C. oryzae*, *P. noxius*, *C. capsici*, *S. cerevisiae*, and *N. crassa* (Figure 2C). Strongest inhibition was observed against *G. boninense* (11.75 ± 0.96 mm) followed by the plant pathogens *C. oryzae* (10 ± 1.41 mm), *P. noxius* (9.75 ± 0.96 mm), and *C. capsici* (8.5 ± 0.58 mm). The weakest inhibition of PHP12 supernatant was against *N. crassa* (5.5 ± 0.58 mm). The inhibition zone of PHP12 against *S. cerevisiae* was the same as *C. capsici* (8.5 ± 0.58 mm). However, five other fungal species including the biocontrol agent *T. asperellum* were not inhibited by the culture supernatant of PHP12 (Figure 2C).

Antifungal activity of PHP12 fractions

Four fractions obtained from precipitation by ammonium sulfate at several concentrations (20, 40, 60 and 80%; henceforth referred to as F2, F4, F6 and F8) were tested for antifungal activity against *S. cerevisiae* (Figure 3A). *Saccharomyces cerevisiae* was used as a model organism due to its fast growth with the antifungal zone being observable in less than 48 h. Fraction F2 showed the highest antifungal activity with inhibition radius at 12.75 ± 0.5 mm. The antifungal activity of the crude fractions decreased following an increase in ammonium sulfate concentration. Leftover supernatant from ammonium sulfate precipitation was also tested for antifungal activity resulting in a clear zone with radius less than 1 mm (data not shown). However, the antifungal activity could be due to the high concentration of ammonium sulfate in the supernatant as we had also observed a similar narrow clear zone from sterile King's B medium added with 80% ammonium sulfate (data not shown).

All of the four fractions were subjected to RP-HPLC. The HPLC chromatogram (Figure 3B) showed that the highest intensity of the peaks was in F2, which eluted at 26 min (labeled as sub-fraction 8). The intensity of the peak decreased following an increase of ammonium sulfate concentration, but it could still be detected in the fraction with 80% ammonium sulfate. It is indicated that although most of the compounds precipitated at 20% (w/v) ammonium sulfate concentration, some of the compounds were still remained in the solution. These remaining compounds precipitated in the subsequent precipitation with ammonium sulfate.

About 14 sub-fractions were collected from RP-HPLC from each of the four main fractions (henceforth labelled as SF1 to SF14; labels such as F2-SF8 will refer to a sub-

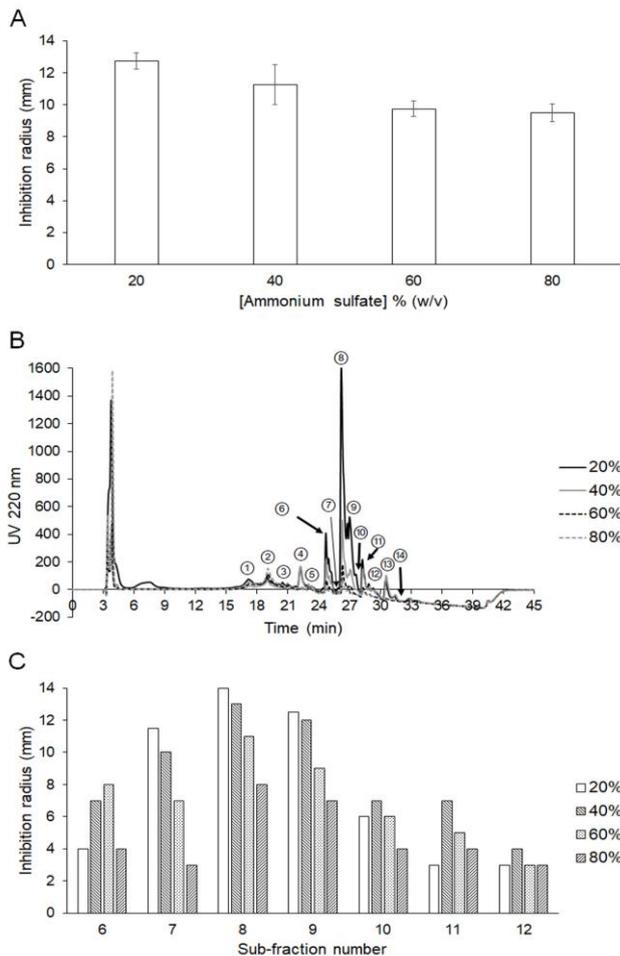


Figure 3: Fractionation of supernatant of PHP12 culture by precipitation with different concentrations of ammonium sulfate (20, 40, 60 and 80% (w/v); designated as F2, F4, F6 and F8 in the text, respectively). (A) Growth inhibition zone in *S. cerevisiae* by antifungal activity of PHP12 crude ammonium sulfate fractions (n=4; error bars are standard errors of the means). (B) RP-HPLC chromatogram of the fractions measured at 220 nm. The circled number refers to the collected sub-fractions. (C). Growth inhibition zone in *S. cerevisiae* by antifungal activity of PHP12 sub-fractions collected from RP-HPLC.

fraction from a specific ammonium sulfate precipitation fraction) resulting in a total of 56 sub-fractions (Figure 3C). All of the 56 sub-fractions were tested for antifungal activity against *S. cerevisiae*, and the result showed that only sub-fractions SF6 to SF12 have antifungal activities. It showed that the antifungal compounds eluted at the same time although they were precipitated at different ammonium sulfate concentrations. SF8 showed the highest antifungal activity than the other sub-fractions (Figure 3C). This trend occurred in SF8 collected from all of the four main fractions (F2 to F8) and indicated that most of the antifungal compounds eluted at 26 min. SF7

to SF9 had a decrease in antifungal activity following an increase in ammonium sulfate concentration. However, the highest antifungal activity in sub-fractions SF10 to SF12 was from the fractions originated from 40% ammonium sulfate concentration (F4). Some sub-fractions (Figure 3C) had higher antifungal activity when compared to the crude fractions (e.g.: F2-SF8 to F2 and F4-SF8 to F4) (Figure 3A) because the sub-fractions were prepared to be more concentrated (20 times) than the crude fractions. Thus, the antifungal activities of sub-fractions collected after RP-HPLC could not be compared directly with the crude fractions from ammonium sulfate precipitation.

Identification of PHP12 antifungal compound

All sub-fractions that showed antifungal activity were subjected to LC-ESI/MS to identify the antifungal compounds of PHP12. Considering that antifungal activities were present in multiple sub-fractions, the target compounds were determined if they were being present conservatively in all sub-fractions and showed different MS intensity in correlation with the antifungal activity of each respective fractions. Based on this hypothesis, a compound with m/z 1211.63 was suspected as the main antifungal compound of PHP12. Due to protonation of the molecule in MS, the molecular weight was assigned as 1210.63 Da (henceforth the compound is referred to as AF-1210). There were no other molecules in all of the sub-fractions, aside from AF-1210, that fulfilled the previously mentioned condition to be suspected as the target antifungal. MS chromatogram of F2-SF6 and F2-SF8 showed the presence of AF-1210 but with different peak intensity (Figure 4A). The peak eluted at 7.51 min and was also detected in other sub-fractions. Similar to sub-fractions F2-SF6 and F2-SF8, peak intensity in the other fractions also varied in correlation with the antifungal activity of each fraction.

Fragmentation of AF-1210 showed a profile similar to that of an oligopeptide. MS/MS profile also showed that AF-1210 is decorated with a sugar monomer and therefore could be a glycopeptide (Figure 4B). The decorated AF-1210 has the m/z 1373.68, which corresponds to the addition of hexose (+162 Da) and was present in all fractions. Interestingly, F2-SF8 also showed a variation of AF-1210 with the molecular weight of 1349 Da (not shown). It is possible that this variant was decorated with a pentose instead of hexose. However, this variant was absent in the other sub-fractions.

Identification of the amino acid sequence of AF-1210 was not as successful as the fragmentation. It resulted in the loss of several residues that did not correspond to the molecular weight of conventional amino acids. This suggested that the compound consisted of modified amino acid that makes identification with MS was difficult. So far, we have identified three amino acid residues, namely glutamine, histidine, and tyrosine (Figure 4B). These amino acids were detected as monomers in the MS profile and thus it was not possible to pinpoint their position in the structure.

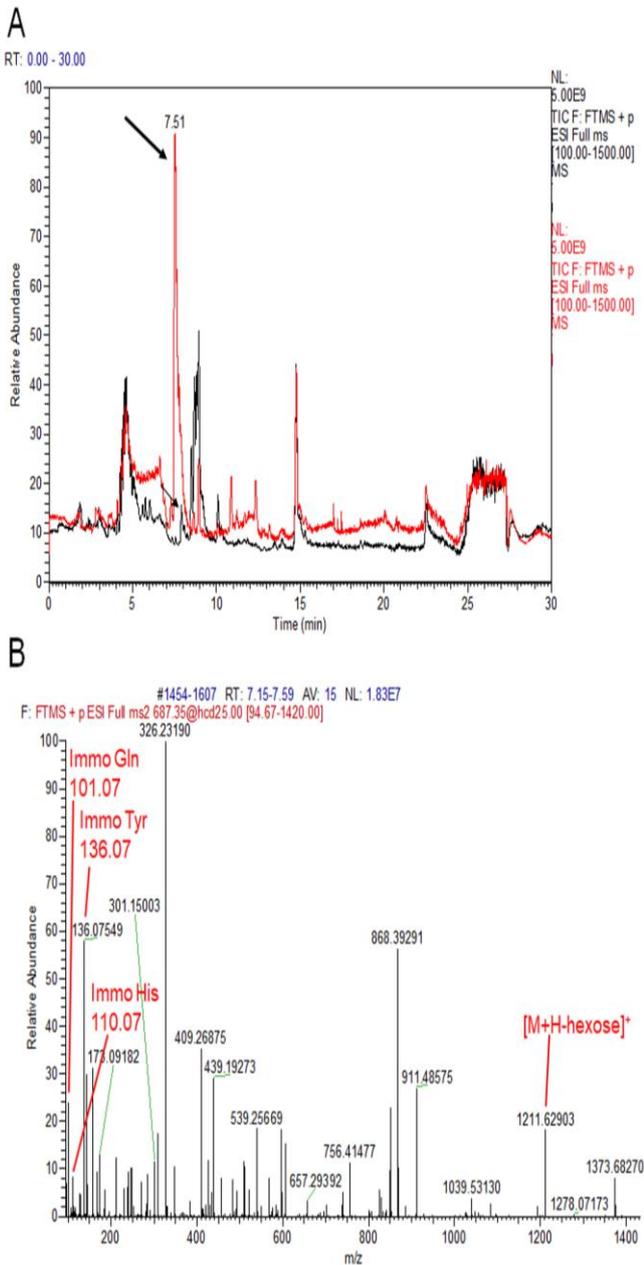


Figure 4: (A) ESI-MS/MS chromatogram of PHP12 sub-fractions #6 (black line) and #8 (red line) from precipitation with ammonium sulfate 20% (w/v) (designated as F2-SF6 and F2-SF8 respectively). Black arrow indicates the presence of suspected antifungal compound AF-1210. (B) ESI-MS/MS fragmentation spectrum of parent ion of AF-1210 (m/z 1373.68). Red annotations refer to aglyconated AF-1210 (m/z 1211.63) and immonium ions of glycine (m/z 101.07), tyrosine (m/z 136.07) and histidine (m/z 110.07).

DISCUSSION

We have characterized a potentially novel antifungal compound that showed strong inhibition against plant pathogenic fungi, particularly *G. boninense*. PHP12 is closely related to *B. stagnalis*. *Burkholderia stagnalis* is a member of Bcc that has only been recently described (De Smet *et al.*, 2015), therefore reports on its characteristics are limited. To the best of our knowledge, this is the first study to evaluate the antifungal activity of *B. stagnalis*. Members of the Bcc group have been reported to be potential as biocontrol and plant growth promoters in agriculture. However, some strains are also known as human pathogenic bacteria, especially in immunocompromised individuals such as people with cystic fibrosis (CF). In the initial description by De Smet *et al.* (2015), strains were isolated from soil, tracheal aspiration of non-CF patient and sputum from CF patient. Strain PHP12 used in this study was isolated from soil, and it is unknown whether this strain is pathogenic or non-pathogenic to human. It has been reported that the overlapping taxonomic designation in *Burkholderia* cannot reflect unambiguously and clearly the division of pathogenic and beneficial strains (De Smet *et al.*, 2015). Pathogenic Bcc strains are distinct from non-pathogenic strains based on fungal-virulence gene loci (Deng *et al.*, 2016). Considering that PHP12 strain will only be applied in agriculture, it is expected not to cause health hazard to humans.

Some species of *Burkholderia* are well-known as antifungal producers. One of the most well-studied antifungal compounds produced by *Burkholderia* is pyrrolnitrin, a derivative of tryptophan. Pyrrolnitrin produced by *B. pyrrocinia* has been used to treat plant diseases caused by *Rhizoctonia solani* (Hill *et al.*, 1994). *B. contaminans* has been reported to produce cyclic oligopeptide, occidiofungin, which was described as decorated with a xylose residue (Lu *et al.*, 2009). Lu *et al.* (2009) also described the presence of novel amino acids that are linked to an acyl chain in *B. contaminans*. Other modifications in occidiofungin include the incorporation of D-amino butyric acid and β -hydroxy modification of tyrosine and asparagine (Gu *et al.*, 2011). The same modifications could contribute to the difficulty in identifying the amino acid sequence of AF-1210 using MS as they could lead to oligopeptide fragments with molecular mass different from conventional amino acids. The result indicated that the compound AF-1210 from PHP12 showed similar characteristic to that of occidiofungin, i.e., the presence of modified amino acids and sugar residues. However, the molecular weight of AF-1210 (1210 Da) was less than occidiofungin (1215 Da). The fragmentation profile of the compound also did not fit with the amino acids that make up the structure of occidiofungin. Based on the MS data, it is possible that AF-1210 to be either a novel compound or a previously undescribed variation of other oligopeptides from *Burkholderia*. An analysis using NMR is necessary to elucidate the structure of AF-1210.

It should be noted that there is a possibility of the existence of other antifungal compounds aside from AF-1210. As previously mentioned, the leftover supernatant derived from precipitation with ammonium sulfate resulted in a narrow clear zone with radius less than 1 mm. We could not ascertain if the clear zone was a result of fungal inhibition by antifungal molecules or not. However, similarly negligible clear zone was also observed from testing sterile King's B medium that contains 80% ammonium sulfate indicating that clear zone was due to a high salt content. However, to properly identify the underlying antifungal mechanism of the supernatant, it first needs to be purified, for example, through dialysis. Considering that *Burkholderia* species are known to produce a wide variety of small antifungal molecules, examination of the leftover supernatant could possibly reveal other antifungal compounds albeit with antifungal activity lower than the AF-1210. However, some molecules, such as pyrrolnitrin, are too small to be precipitated using ammonium sulfate and will be left behind in the supernatant. Therefore, such molecules might have been excluded from our analysis. But given that AF-1210 is definitely the compound showed in MS and has strong antifungal, whereas the possibly antifungal activity of leftover supernatant is negligible, we conclude that AF-1210 is the most compelling candidate for the antifungal compound in PHP12.

Unfortunately, further purification of the antifungal sub-fraction using HPLC was not successful. An attempt to purify the compound to yield a more definitive data from LC-ESI/MS using longer column and size exclusion chromatography (SEC) was also not successful because the peaks could not be separated. It might be possible to obtain a purer fraction using UPLC equipped with a fraction collector. Further purification using SEC followed by preparative HPLC was proposed by Ross *et al.* (2014) to purify antifungal polyketides from *B. gladioli*. The same approach could be adapted considering the possibility that similar polyketides might also be produced by PHP12. The method described by Ross *et al.* (2014) would also allow the fraction to be further characterized using NMR since the method used in our experiment resulted in a fraction that still contained background contaminants which can hinder NMR analysis.

The tolerance of *T. asperellum* towards PHP12 antifungals showed the potential of PHP12 bacterial strain as a candidate for biocontrol agent against plant fungal pathogens. Some members of genus *Trichoderma* are known to be capable of inhibiting the growth of fungal pathogens such as *G. boninense* and *P. noxius* (Angel *et al.*, 2016; Kwan *et al.*, 2016). Schwarze *et al.* (2012) reported that *Trichoderma* sp. can reduce the rate of wood decomposition from white rot disease caused by *P. noxius*. On the other hand, there are also several reports on the ability of bacteria in inhibiting *P. noxius*. Several strains of actinomycetes showed antagonistic activity against *P. noxius* through the production of chitinase (Yanti *et al.*, 2012). In this study, we showed that PHP12 was able to inhibit the growth of *P. noxius*.

Bacterial species such as *Burkholderia* and *Bacillus* can inhibit *G. boninense* (Buana *et al.*, 2014; Pramudito *et al.*, 2018). Moreover, Alexander *et al.* (2015) stated that *Bacillus* sp. has synergistic activity with *Trichoderma* sp. in inhibiting *G. boninense*. The synergism is due to the presence of cell wall degrading enzymes produced by fungi (*Trichoderma* sp.), which in turn increases the accessibility of bacterial antifungal compounds (Woo *et al.*, 2002). Further research is needed to investigate the possible synergistic mechanism between PHP12 and *Trichoderma* sp.

CONCLUSION

In conclusion, this study showed that *B. stagnalis* PHP12 has a potential to be used as a biocontrol agent as it is capable of inhibiting pathogenic fungal strains but not the beneficial ones. The compound was suspected to be a small oligopeptide but further elucidation of its structure was found to be difficult due to modification of the amino acid building blocks. Nevertheless, PHP12 can therefore be used or tested for field application in the plantation industry. We hope that our findings would initiate further research on the antifungal activity of *B. stagnalis* especially on strains related to PHP12.

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REFERENCES

- Aderungboye, F. O. (1977). Diseases of the oil palm. *Proceedings of the National Academy of Sciences of the United States of America* **23**(3), 305-326.
- Agrios, G. (2005). Plant Pathology. 5th Edn. Academic Press. Cambridge, MA, USA. pp. 1-952.
- Alexander, A., Dayou, J. and Chong, K. -P. (2015). Morphological changes of *Ganoderma boninense* mycelia after challenged by *Trichoderma* and *Bacillus*. *AIP Conference Proceedings* **1699**(1), 020075.
- Angel, L. P. L., Yusof, M. T., Ismail, I. S., Ping, B. T. Y., Azni, I. N. A. M., Kamarudin, N. H. and Sundram, S. (2016). An *in vitro* study of the antifungal activity of *Trichoderma virens* 7b and a profile of its non-polar antifungal components released against *Ganoderma boninense*. *Journal of Microbiology* **54**, 732-744.
- Buana, R. F. N., Wahyudi, A. T. and Toruan-Mathius, N. (2014). Control activity of potential antifungal-producing *Burkholderia* sp. in suppressing *Ganoderma boninense* growth in oil palm. *Asian Journal Agricultural Research* **8**, 259-268.
- Cordova-Kreylos, A. L., Fernandez, L. E., Koivunen, M., Yang, A., Flor-Weiler, L. and Marrone, P. G. (2013). Isolation and characterization of *Burkholderia rinojensis* sp. nov., a non-*Burkholderia cepacia* complex soil bacterium with insecticidal and mitocidal

- activities. *Applied and Environmental Microbiology* **79**, 7669-7678.
- Darma, R., Purnamasari, M. I., Agustina, D., Pramudito, T. E., Sugiharti, M. and Suwanto, A. (2016). A strong antifungal-producing bacteria from bamboo powder for biocontrol of *Sclerotium rolfsii* in melon (*Cucumis melo* var. *amanta*). *Journal of Plant Pathology and Microbiology* **7**, 334.
- De Smet, B., Mayo, M., Peeters, C., Zlosnik, J. E. A., Spilker, T., Hird, T. J., LiPuma, J. J., Kidd, T. J., Kaestli, M., Ginther, J. L., Wagner, D. M., Keim, P., Bel, S. C., Jacobs, J. A., Currie, B. J. and Vandamme P. (2015). *Burkholderia stagnalis* sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. *International Journal of Systematic and Evolutionary Microbiology* **65**, 2265-2271.
- Deng, P., Wang, X., Baird, S. M., Showmaker, K. C., Smith, L., Peterson, D. G. and Lu, S. (2016). Comparative genome-wide analysis reveals that *Burkholderia contaminans* MS14 possesses multiple antimicrobial biosynthesis genes but not major genetic loci required for pathogenesis. *Microbiology Open* **5**, 353-369.
- Ghisalberti, E. L. and Rowland, C. Y. (2004). Antifungal metabolites from *Trichoderma harzianum*. *Journal of Natural Products* **56**(10), 1799-1804.
- Glick, B. R. and Bashan, Y. (1997). Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. *Biotechnology Advances* **15**, 353-378.
- Gu, G., Smith, L., Liu, A. and Lu, S. -E. (2011). Genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. *Applied and Environmental Microbiology* **77**, 6189-6198.
- Hill, D. S., Stein, J. I., Torkewitz, N. R., Morse, A. M., Howell, C. R., Pachlatko, J. P., Becker, J. O. and Ligon, J. M. (1994). Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. *Applied and Environmental Microbiology* **60**, 78-85.
- Hwang, J., Chilton, W. S. and Benson, D. M. (2002). Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biological Control* **25**, 56-63.
- Katoch, M. and Pull, S. (2017). Endophytic fungi associated with *Monarda citriodora*, an aromatic and medicinal plant and their biocontrol potential. *Pharmaceutical Biology* **55**, 1528-1535.
- Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547-1549.
- Kwan, H. S., Bas, M. C., Schwarze, F. W. M. R., Chu, L. M., Lam, R. Y. C., Tang, A. M. C., Ribera, J., Schubert, M. and Leung, M. W. K. (2016). *In-vitro* evaluation of antagonistic *Trichoderma* strains for eradicating *Phellinus noxius* in colonised wood. *Journal of Tropical Forest Science* **28**, 457-468.
- Lu, S. -E., Novak, J., Austin, F. W., Gu, G., Ellis, D., Kirk, M., Wilson-Stanford, S., Tonelli, M. and Smith L. (2009). Occidiofungin, a unique antifungal glycopeptide produced by a strain of *Burkholderia contaminans*. *Biochemistry (Moscow)* **48**, 8312-8321.
- Majeed, R. A., Shahid, A. A., Ashfaq, M., Saleem M. Z. and Haider, M. S. (2015). First report of *Curvularia lunata* causing brown leaf spots of rice in Punjab, Pakistan. *Plant Disease* **100**(1), 219.
- Maznah, Z., Halimah, M., Ismail, S. and Idris, A. S. (2015). Dissipation of the fungicide hexaconazole in oil palm plantation. *Environmental Science and Pollution Research* **22**, 19648-19657.
- Omamor, I. B., Asemota, A. O., Eke, C. R. and Eziashi, E. I. (2007). Fungal contaminants of the oil palm tissue culture in Nigerian institute for oil palm research (NIFOR). *African Journal of Agricultural Research* **2**, 534-537.
- Pramudito, T. E., Agustina, D., Nguyen, T. K. N. and Suwanto, A. (2018). A novel variant of narrow-spectrum antifungal bacterial lipopeptides that strongly inhibit *Ganoderma boninense*. *Probiotics and Antimicrobial Proteins* **10**, 110-117.
- Romero, D., Vicente, A. D., Olmos, J. L., Dávila J. C. and Pérez-García, A. (2007). Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podospaera fusca*. *Journal of Applied Microbiology* **103**, 969-976.
- Ross, C., Opel, V., Scherlach, K. and Hertweck, C. (2014). Biosynthesis of antifungal and antibacterial polyketides by *Burkholderia gladioli* in coculture with *Rhizopus microsporus*. *Mycoses* **57**, 48-55.
- Schwarze, F. W. M. R., Jauss, F., Spencer, C., Hallam, C. and Schubert, M. (2012). Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. *Biological Control* **61**, 160-168.
- Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512-526.
- Than, P. P., Prihastuti, H., Phoulivong, S., Taylor, P. W. J. and Hyde, K. D. (2008). Chilli anthracnose disease caused by *Colletotrichum* species. *Journal of Zhejiang University Science B* **9**, 764-778.
- Woo, S., Fogliano, V., Scala, F. and Lorito, M. (2002). Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie Van Leeuwenhoek* **81**, 353-356.
- Yanti, Y., Zainon, M. N. and Marshida, A. H. U. (2012). Antagonistic effects of actinomycetes towards plant pathogen *Phellinus noxius*. In: 2012 IEEE Colloquium on Humanities, Science and Engineering (CHUSER). 2012 IEEE Colloquium on Humanities, Science and Engineering Research, Magellan Sutura Resort, Kota Kinabalu, Sabah, Malaysia. pp. 419-423.