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PROFILING OF ANTI-FUNGAL ACTIVITY OF *Trichoderma virens* 159C INVOLVED IN BIOCONTROL ASSAY OF *Ganoderma boninense*

LEE PEI LEE ANGEL*; SHAMALA SUNDRAM*; BONNIE TAY YEN PING*; MOHD TERMIZI YUSOF and INTAN SAFINAR ISMAIL‡**

ABSTRACT

Trichoderma *has long been recognised as a potential biological control agent (BCA) against pathogenic fungi due to antagonistic characteristics and it has successfully controlled* Ganoderma boninense *at the nursery stage in previous study. This study attempts to identify the mechanisms involved in the suppression of* G. boninense *and the anti-fungal compounds released by endophytic* Trichoderma virens *159c. Therefore, culture filtrates of endophytic* T. virens *159c was extracted using hexane, ethyl acetate (EtOAc) and butanol (BuOH) and anti-fungal activity was tested. The EtOAc extract showed highest anti-fungal activity with percentage inhibition of radial growth (PIRG) of 78.39% ±5.40. The scanning electron microscope (SEM) showed severe deformation of* G. boninense *PER 71 mycelia observed at the inhibition region caused by EtOAc extract. Further fractionated with column chromatography and anti-fungal assay revealed that fractions 2 and 4 had highest anti-fungal activity. Analysis carried out using gas chromatography-mass spectrometry detector of the active fraction allowed the identification of acetamide, alcohol, lactones and free fatty acids. Phenylethyl alcohol (PEA) was discovered as a unique compound because the presence was only in the highly inhibitory fraction of* T. virens*. In addition,* dl-*mevalonic acid lactone in fraction 4 of* T. virens *159c was first reported in* T. virens.

Keywords: endophytes, anti-fungal, *Trichoderma virens*, biological control agent, *Ganoderma boninense*.

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INTRODUCTION

Biological control agent (BCA) is defined as the use of a single or mixed culture of organisms to control phytopathogens and to allow the restoration of some regulating factors that limits the competitive ability of pathogens (Chet and Inbar, 1994). Antibiosis is an important attribute of BCA which produces both secondary metabolites and enzymes that are responsible for the suppression of various pathogens ranging from bacteria to fungi (Whipps, 1997). There are 1500 compounds of fungal metabolites which have been identified from various fungi, and more than half of these are found to be anti-bacterial, anti-tumour or anti-fungal metabolites (Keller *et al*., 2005; Derntl *et al*., 2017). Anti-fungal agents are known to have their own mechanisms of action in disrupting the metabolism of pathogens (Ghannoum and Rice, 1999; Johann *et al*., 2012). Due to these properties, anti-fungal compounds are of major interest to

* Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.
E-mail: shamala@mpob.gov.my

** Department of Microbiology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

‡ Department of Chemistry, Faculty of Science;
Universiti Putra Malaysia, 43400 UPM Serdang,
Selangor, Malaysia.

be explored further. Antibiosis is a widespread strategy used by *Trichoderma* for defence against plant pathogens (Hjeljord and Tronsmo, 1998). The role of these secondary metabolites is revealed as being important compounds that lead to the success of antibiosis (Vinale *et al*., 2006; 2008; Reino *et al*., 2007; El-Hasan *et al*., 2009). Recently, fungal endophytes are recognised as prolific producers of structurally unique and biologically active natural secondary metabolites with excellent mechanisms of antibiosis (Kusari *et al*., 2012; Zhang *et al*., 2013). Iron acquisition is an important mechanisms for BCA as iron is an essential element in metabolic and informational cellular pathways for most organisms (Howard, 1999; Symeonidis and Marangos, 2012). It is usually aided by the synthesising low molecular weight iron chelators by most microorganisms under conditions of iron starvation known as siderophore (Miethke and Marahiel, 2007) and can be detected with chrome azurol S (CAS) agar (Schwyn and Neilands, 1987). Therefore, the battle between the pathogen and the BCA for iron acquisition has been an interesting interaction as both microorganisms require the element for survival. Sundram (2013) isolated endophytic *Trichoderma* from oil palm roots and investigated its anti-fungal activity against *Ganoderma boninense*. In addition, nursery and field trials conducted by Sundram *et al*. (2016) showed significant *Ganoderma* disease suppression after application of these endophytic *T. virens*. *G. boninense* is a pathogen that caused basal stem rot disease (BSR) to oil palm. BSR disease is currently a major threat to the oil palm industry in Malaysia that leads to serious economic losses (Fee, 2011). As a matter of fact, there are several species of *Ganoderma* involved in BSR infection but *G. boninense* was reported to be the most aggressive compared to the others (Idris, 1999). Previously, Angel *et al*. (2016) studied the activity of the potential endophytic *T. virens* 7b against *G. boninense* and a notable discovery is the secretion of phenylethyl alcohol (PEA) by *T. virens*, which has not been reported previously. Hence, the present investigation aims to evaluate the chemical and physical mechanism of another potential endophytic strain isolated from oil palm roots, *T. virens* 159c against *G. boninense* and identify the compounds responsible in suppressing the pathogen's growth.

METHODOLOGY

Source of Endophytic *Trichoderma*

The endophytic isolate, *T. virens* 159c, was supplied by *Ganoderma* and Diseases Research for Oil Palm (GanoDROP) Unit, Malaysian Palm Oil Board (MPOB), Malaysia and selected based on two screening methods; dual culture and poison agar technique (Sundram, 2013). The isolate was maintained at 28°C in potato dextrose agar (PDA) (Difco, France) at dark. *Trichoderma* strain was characterised and identified using ITS primers: TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (Howlett *et al*., 1992). ITS regions that have been used to distinguish the taxa between fungal isolates, hence ITS-based markers will be carried out for identification process (Badotti *et al*., 2017). *G. boninense* PER 71, pathogen used for this study was isolated by Idris (1999) from Teluk Intan, Perak, Malaysia and was maintained with PDA at 28°C in the dark.

Siderophore Detection of *Trichoderma virens* 159c

Detection of the production of siderophore by *T. virens* 159c was done as described by Milagres *et al*. (1999). Petri dishes containing 25 ml of 2% malt extract agar (MEA) were prepared. The medium was cut into half after it solidified and was replaced by CAS-blue agar. The halves containing MEA was inoculated with *T. virens* 159c. The inoculum was placed as far as possible from the borderline between the two media. The plates were incubated at 28ºC for three weeks. The rate of colour changes on CAS-blue agar by *T. virens* 159c were monitored and recorded. All experiments were conducted in five replicates.

Culture Filtrate Cultivation and Solvent Extraction

The methodology was adapted from Angel *et al*. (2016). Dried hexane, ethyl acetate (EtOAc), butanol (BuOH) extracts of *T. virens* 159c and crude *T. virens* 159c extracts were obtained from the extraction and the dried extract was maintained at -30˚C.

Anti-fungal Properties of *T. virens* 159c Extracts towards *Ganoderma boninense*

The effects of extracts with increasing polarity were determined with assay described by Angel *et al*. (2016) previously. Benlate®, a chemical fungicide act as a positive control. The anti-fungal assay was performed using five replicates. The plates were incubated at 28°C, and *G. boninense* PER 71 growth was monitored after 1, 3, 5, 7 and 8 days. The PIRG of *G. boninense* PER 71 on Day 7 was calculated.

Observation of Mycelia Using a Scanning Electron Microscope (SEM)

Mycelial discs with healthy *G. boninense* PER 71 that were subjected to different treatments using *T. virens* 159c extracts (hexane, EtOAc, and BuOH extracts) and mycelia disc subjected to positive control (Benlate®) were prepared. Preparation of

Ganoderma mycelia for SEM was based on Alves *et al*. (2013) and Angel *et al*. (2016). The morphology of each *G. boninense* mycelia were observed under SEM.

Isolation of Fractions from *Trichoderma virens* 159c EtOAc Extract by Column Chromatography

The *in vitro* bioassay revealed that the EtOAc fraction of *T. virens* 159c had the most significant anti-fungal activity against *G. boninense* PER 71. Therefore, this fraction was selected for further purification. The EtOAc extract was subjected to column chromatography over silica gel (Merck, 230-400 mesh, Germany) for the separation of different constituents using a mixture of solvents: DCM (Qrec, Thailand), EtOAc (Qrec, Thailand) and ethanol (EtOH) (Qrec, Thailand). The column, packed with silica gel 15 cm x 3.5 cm, was eluted with DCM, DCM-EtOAc, EtOAc-EtOH in increasing order of polarities to obtain a total of 13 fractions by collecting 200 ml of each fraction. The isolated fraction was evaporated to dryness with a rotary evaporator at 60°C. The dried extract was measured and kept at -30°C.

Anti-fungal Activity of Fractionated Extract from *Trichoderma virens*

The dried extract weight was measured and it was dissolved in dimethyl sulphoxide (DMSO) at 10 mg ml^{-1}. Thirteen fractions from the column chromatography were tested for anti-fungal activity. The anti-fungal activities of each fraction against *G. boninense* PER 71 were determined using a modified well diffusion assay (Angel *et al*., 2016). The anti-fungal assay was performed with five replicates. The plates were incubated at 28ºC, and *G. boninense* PER 71 growth was monitored for four days. The inhibition zone distance was calculated by measuring the distance from both sides of mycelium end towards the well.

Identifying the Bioactive Compounds in the Active Fraction Using a Gas Chromatography-Mass Spectrometry Detector (GC-MSD)

The active fractions, fraction 2 (170.6 mg) and fraction 4 (51.8 mg) obtained from column chromatography were dissolved in a mixture of EtOAc and methanol (1:1; v:v) to 10 mg ml^{-1}. The samples were injected to GC-MSD (Agilent Technologies 7890A, USA) equipped with the non-polar capillary column HP5 (30 m length x 0.25 mm i.d., 0.25 μm film thickness) and helium (99% purity) as the carrier gas at a 2 ml min^{-1} flow rate. The method was adapted from Angel *et al*. (2016). The compounds were identified based on matching between the mass spectra (MS) of unknown and reference compounds in the National Institute of Standards and Technology (NIST) library.

Statistical Analysis

The statistical analyses were performed using the Statistical Package of Social Science (SPSS). Tukey's test at $P < 0.05$ was applied to determine whether differences between treatments were significant. All the data was arcsine transformed before added to the statistical analysis.

RESULTS

Identifying Endophytic *Trichoderma*

Based on the polymerase chain reaction (PCR) analyses, the ITS region of the *T. virens* 159c was amplified at 700 - 750 bp. The sequence analysis for the *T. virens* 159c isolate exhibited 99% identity to the *Trichoderma virens* strain isolated from soil matched through NCBI blasting. The sequence data for *T. virens* 159c was submitted to the National Institute of Standards and Technology (NCBI) and given accession numbers KT363921 (*T. virens* 159c).

Efficiency of Siderophore Production by *Trichoderma virens* 159c

The siderophore production of the antagonistic fungal strains *T. virens* 159c was observed based on the colour changes in the CAS-blue agar. *Figure 1a* shows that the control CAS-blue agar plate remained blue even after 21 days of incubation. *T. virens* 159c only required three days to reach the border of the plate despite insufficient iron content in 2% MEA. There was rapid change of the CAS-blue agar since most of the agar turned yellow in 14 days (*Figure 1b*) and completely yellow in less than 21 days (*Figure 1c*).

Efficacy of Fractionated Culture Filtrates of *T. virens* 159c in the Inhibition of *Ganoderma boninense*

Table 1 shows the PIRG of *G. boninense* PER 71 when tested with different extracts of *T. virens* 159c. The results showed that EtOAc extract (78.39% ±5.40) gave the best suppression of *G. boninense* PER 71 as compared to the other two extracts. In addition, the EtOAc extract's performance was comparable with Benlate® which gave a percentage inhibition of radial growth (PIRG) of 79.88% ±2.80 with no significance difference. This also provides information that the anti-fungal activity is mostly contributed by a mixture of polar and non-polar compounds. *Figure 2* illustrates the suppression on *G. boninense* PER 71 by the different extracts and EtOAc extracts (*Figure 2d*) showed highest inhibition towards *G. boninense* PER 71.

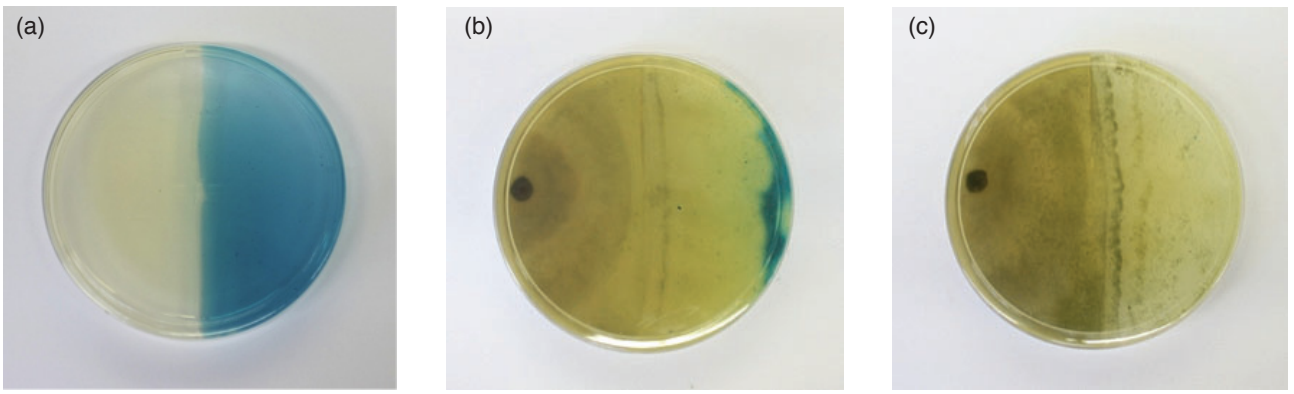

Figure 1. Trichoderma virens *159c siderophore detection using the chrome azurol S (CAS) plate agar assay; (a) CAS-blue agar without presence of* T. virens *159c (control); (b)* T. virens *159c incubated for 14 days; (c)* T. virens *159c incubated for 21 days.*

Observation of *G. boninense* Mycelia Using SEM

The mycelia subjected to blank DMSO as negative control was presented in a normal and intact tubular shape with smooth cell wall (*Figure 3a*). However, prominent changes of the *G. boninense* hyphae were found after subjected to crude culture filtrate extract. There were clumping and irregular swelling of the mycelia as seen in *Figure 3b* in which causing the morphological defect on the mycelia of *G. boninense*. The detached and broken mycelia characteristics caused by EtOAc extract of *T. virens* 159c (*Figure 3c*) caused the mycelia network in an inorganised manner. However, a different hyphal morphology was observed when subjected to Benlate® (*Figure 3d*) when compared with the EtOAc extract where the deformation of hyphae was mainly clumping.

TABLE 1. INHIBITION OF *Ganoderma boninense* BY CHEMICAL AND CULTURE FILTRATES OF *Trichoderma virens* 159c*

Type of extract	Percentage inhibition of radial growth, %
Crude	44.31 ± 7.84a
Hexane	21.85 ± 3.40b
Ethyl acetate	78.39 ± 5.40b
Butanol	2.56 ± 4.49c
Benlate® (Positive control)	79.88 ± 2.80d

Note: *Percentage of inhibition growth was calculated using blank dimethyl sulphoxide (DMSO) as a negative control. Each value is the mean percentage of three experiments ± standard deviation. Means within the same column followed by the superscript of same letters are not significantly different at P <0.05 with Tukey's test.

Screening of the Anti-fungal Activity for Each Column Eluted Fraction against *G. boninense*

EtOAc extraction yielded higher amount of extracts compared to hexane extraction. The column separation resulted in 13 fractions which were subjected for screening against the pathogen. The anti-fungal assay showed fractions 2, 3, 4, 5, 6, 7, and 8 were highly inhibitory against *G. boninense* PER 71 (*Figure 4*) with inhibition at 23.4 mm, 22.7 mm, 24.2 mm, 21.5 mm, 22.4 mm, 23.7 mm, and 22.3 mm, respectively after four days of incubation. The inhibition by crude EtOAc extract from *T. virens* 159c (21.9 mm) was equally effective as the fractionated

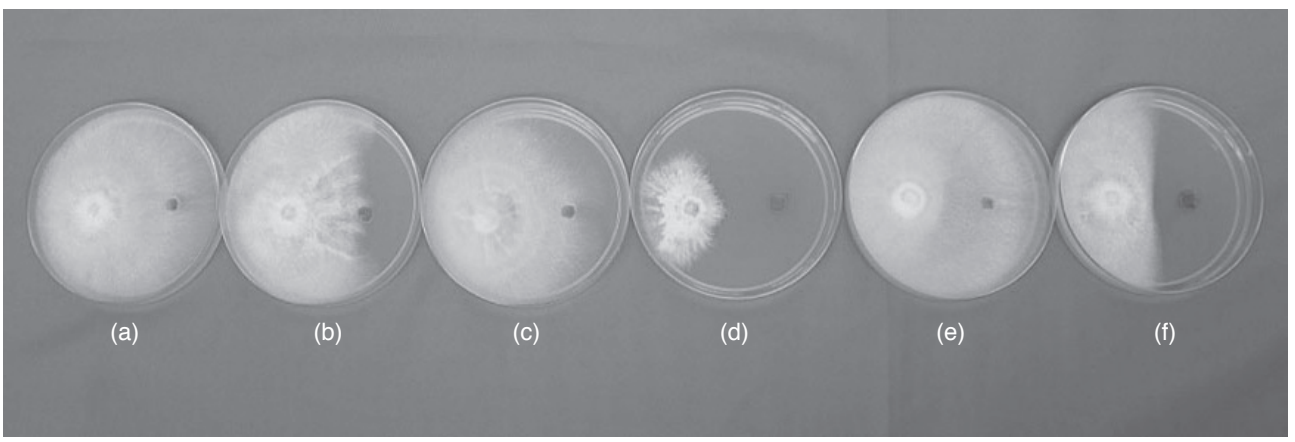

Figure 2. Effect of crude and fractionated Trichoderma virens *159c extract against* Ganoderma boninense; *(a) negative control; (b) crude extract; (c) hexane extract; (d) ethyl acetate (EtOAc) extract; (e) butanol (BuOH) extract; (f) Benlate®.*

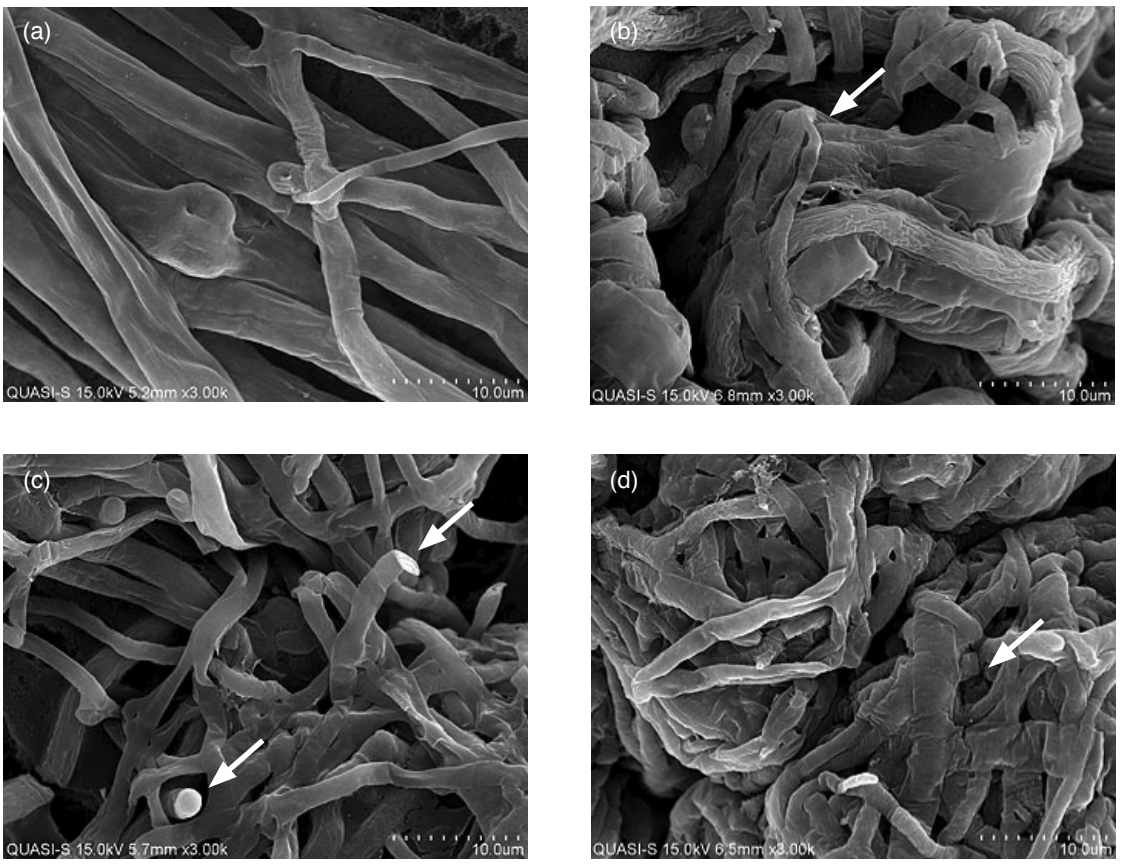

Figure 3. The effects of the extract of Trichoderma virens *159c on mycelium of* Ganoderma boninense *PER 71 viewed under scanning electron microscope (SEM) (10 µm); (a) negative control; (b) crude extract; (c) ethyl acetate (EtOAc) extract; (d) Benlate ®. Arrow showing abnormalities on the* Ganoderma *hyphae.*

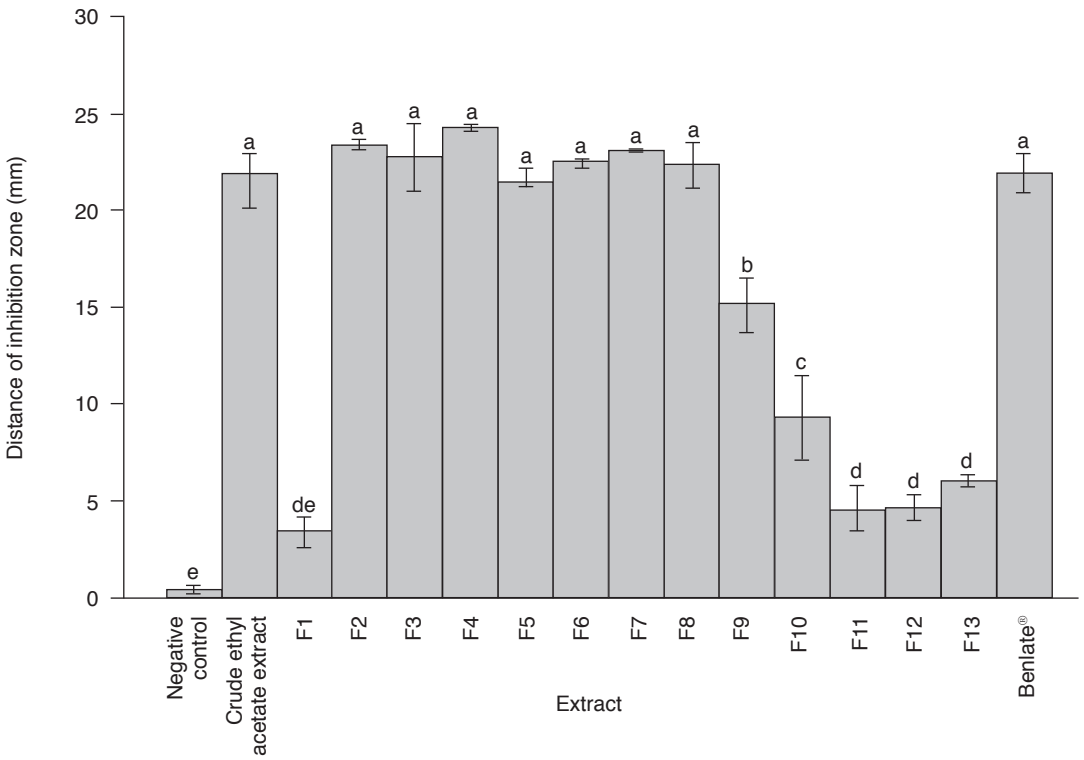

Figure 4. Distances of inhibition zones (mm) of fractions (F) of Trichoderma virens *159c ethyl acetate (EtOAc) extract. Blank dimethyl sulphoxide (DMSO) as negative control. Each value is the mean percentage of three experiments ± standard deviation. Means within the same column with the same superscript letters are not significantly different at P<0.05 with Tukey's test.*

extracts with no significant difference with all seven active extracts. The *G. boninense* PER 71 was found healthy in the negative control plate after four days of incubation. The efficiency of the active extracts from *T. virens* 159c was further confirmed as the activity was not significant with the anti-fungal activity caused by Benlate®. However, fractions 2 and 4 showed the highest inhibitions among the other active extracts and might have consisted of anti-fungal compounds that highly suppressed the growth of *G. boninense* PER 71. The two fractions were subjected for further evaluation.

Identification of Semi-polar Compounds Produced by *T. virens* 159c with GC-MSD

The results presented in *Tables 2* and *3* were profiles of compounds in active fractions 2 and fraction 4 of *T. virens* 159c that were possibly responsible for the inhibition of *G. boninense* PER 71. The chromatograms are shown in *Figure 5*. A total of 14 compounds in fraction 2 (*Table 2*), nine compounds in fraction 4 (*Table 3*) were identified using the NIST library with a probability matching greater than 50%.

DISCUSSION

The ability to compete for nutrients with pathogenic fungi is a key mechanism that allows the antagonistic fungi to become a successful biocontrol agent. In this study, the ability of *T. virens* 159c and *G. boninense* in the release of siderophores (a chelator ions) for the uptake of iron was studied by using CAS assay, which is generally used method to detect the siderophore by microorganisms (Schwyn and Neilands 1987). The observation showed that *T. virens* 159c being superior producer of siderophore when compared to the endophytic *T. virens* 7b reported by Angel *et al.* (2016). Differences in the discolouration of the CAS-blue agar showed that different types of siderophore

TABLE 2. METABOLITES IDENTIFIED FROM FRACTION 2 OF *Trichoderma virens* 159c ETHYL ACETATE (EtOAc) EXTRACT USING A GAS CHROMATOGRAPHY-MASS SPECTROMETRY DETECTOR (GC-MSD)

No.	Metabolites	Retention time (min)	Matching of spectra (%)	Percentage composition* (%)
1	Cyclopentanol, 1-methyl	4.306	80.3	7.7
2	Cyclopentanol, 3-methyl	5.526	56.3	1.7
3	Unknown	7.194	-	10.9
4	Unknown	7.398	-	3.8
5	Unknown	7.906	-	8.5
6	3,4-dimethylpent-2-en-1-ol	8.516	87.7	3.1
7	Unknown	9.072	-	11.1
8	Unknown	9.291	-	46.8
9	Unknown	10.147	-	2.3
10	Unknown	11.607	-	1.4
11	Phenylethyl alcohol	13.752	87.3	1.9
12	Acetamide, *N*-(2-phenylethyl)	24.545	82.3	0.2
13	Dodecanoic acid	25.738	79.0	0.1
14	Pyrrolo (1,2a) pyrazine, 1,4-dione hexahydro-3-(2 methylpropyl)	33.520	85.9	0.5

Note: *Percentage composition based on peak height.

TABLE 3. METABOLITES IDENTIFIED FROM FRACTION 4 OF *Trichoderma virens* 159c ETHYL ACETATE (EtOAc) EXTRACT USING A GAS CHROMATOGRAPHY-MASS SPECTROMETRY DETECTOR (GC-MSD)

No.	Metabolites	Retention time (min)	Matching of spectra (%)	Percentage composition* (%)
1	Cyclopentanol, 1-methyl	4.317	50.0	3.8
2	1,3-Octanediol	6.815	51.4	0.9
3	*dl*-mevalonic acid lactone	18.036	93.9	12.4
4	Unknown	18.630	-	0.9
5	Unknown	20.325	-	3.1
6	Benzeneethanol, 4-hydroxy	22.545	74.0	6.6
7	Unknown	23.727	-	1.5
8	Unknown	25.749	-	3.5
9	Tetradecanoic acid	30.188	81.4	67.1

Note: * Percentage composition based on peak height.

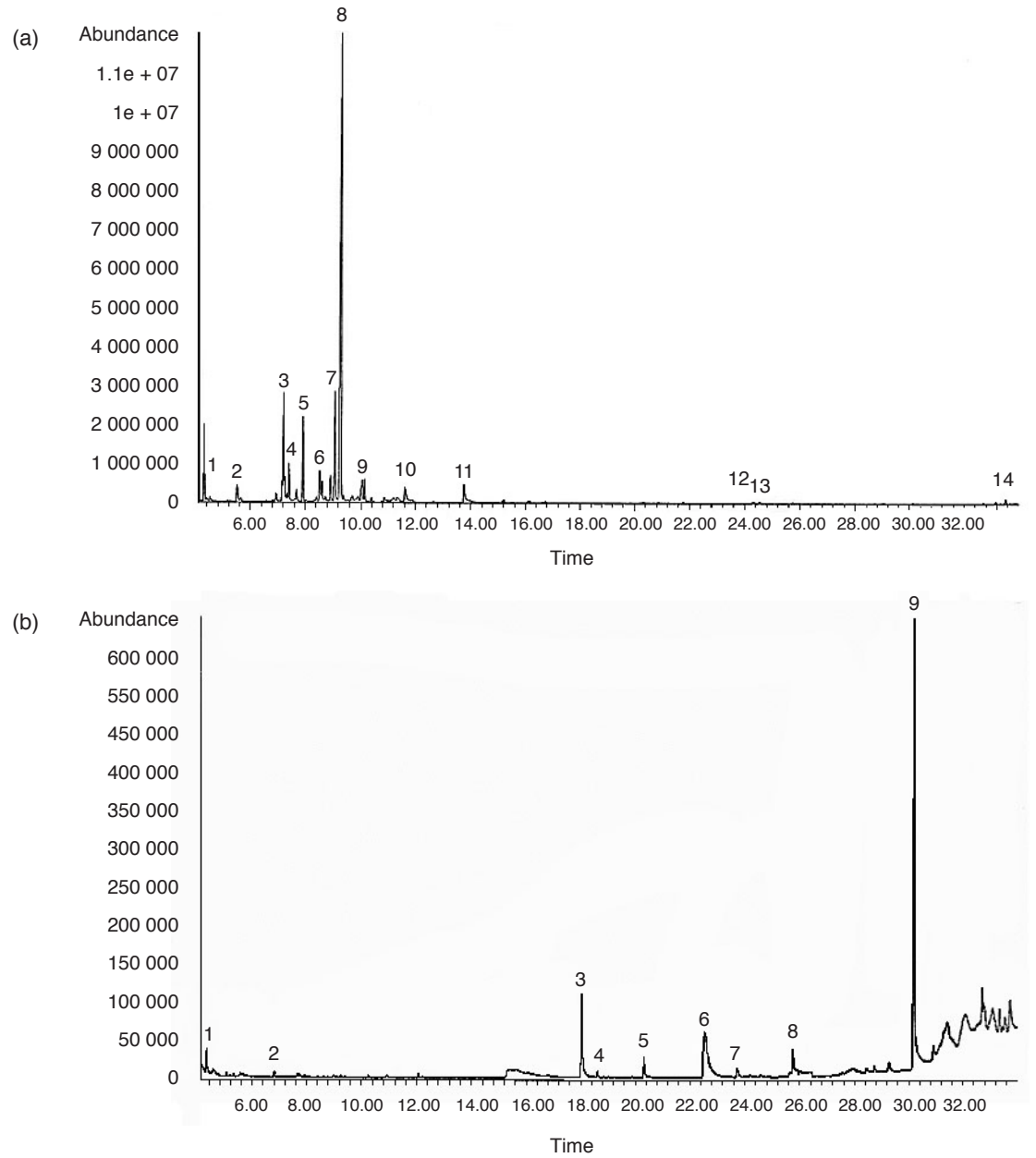

Figure 5. Gas chromatography-mass spectrometry detector (GC-MSD) profiles of (a) fraction 2 and (b) fraction 4 ethyl acetate (EtOAc) extract from Trichoderma virens *159c.*

were being released (Miethke and Marahiel, 2007). It was observed that *T. virens* 159c secreted the hydroxamate type of siderophore similar to *T. virens* 7b reported previously. *Trichoderma* were reported as hydroxamate producers and hydroxamate siderophores possessed higher stability compared to other types of siderophores which may be a factor for their ecological predominance (Helm and Winkelmann, 1994; Wilhite *et al*., 2001). On the contrary, *G. boninense* PER 71 produced siderophores at a much slower rate which showed that the superiority of the endophytic *Trichoderma* in iron acquisition (Angel *et al*., 2016). This phenomenon left *G. boninense* PER 71 in the iron starvation mode. The rate of siderophore production for a potential BCA is very important in the competition for nutrients with the pathogens, as it creates iron starvation in the environment by acquiring the available nutrient for survival (Loper and Buyer, 1991). This study revealed that *T. virens* 159c is able to utilise a high affinity iron transport system and efficiently produce siderophores to compete under iron deficient conditions.

In the present work, the secondary metabolites released from *T. virens* 159c were characterised to identify the potential compounds that are responsible in the inhibition of *G. boninense* PER 71. Usage of solvents with different polarities allowed discrimination between non-polar and polar fraction activities (Mahlke *et al.,* 2009). The EtOAc extract

of *T. virens* 159c effectively inhibited the activity of *G. boninense* PER 71 which might be due to the enrichment of anti-fungal compounds in the EtOAc extract without any interference of the other non-active compound from the other fractions (Krishna Reddy *et al.,* 2009). These findings are in agreement with various studies that was carried out in evaluating the anti-fungal properties of *Trichoderma* using EtOAc extraction (El-Hasan *et al*., 2009; Vinale *et al*., 2009), showing greater biological activity in semi-polar rather than other polarities. However, different *Trichoderma* strains produced different types of secondary metabolites in order to defend against the pathogenic fungi (Jeleń *et al*., 2013). Although both endophytic *Trichoderma* (*T. virens* 7b and 159c) were identified as *T. virens*, distinct characteristics were observed. The growth pattern for both *Trichoderma* were different on PDA plate and the colour changed of potato dextrose broth (PDB) after incubation of *T. virens* 159c for seven days was lighter compared to *T. virens* 7b. Different results were observed as hexane extract of *T. virens* 7b possessed higher inhibition effect towards *G. boninense* PER 71 instead of ethyl acetate extract (Angel *et al*., 2016). This explains the production of metabolites is strain dependent, whereby different strain of same species can produce different types of metabolites with different effects on target pathogens (Bailey *et al*., 2010). Therefore, both strains showed different anti-fungal activities and produces different metabolites compounds.

Deformation of hyphae caused by EtOAc extract observed in the study confirmed the presence of anti-fungal agents in the active extract that was responsible for the disturbance of *G. boninense* PER71 growth (Mares *et al*., 2004; Kim *et al*., 2012). This was achieved due to the biosynthesis of a wide array of secondary metabolites (Reino *et al*., 2007). Although there was a slight difference in the form of damage caused by Benlate®, EtOAc extract from *T. virens* 159c still possessed unique mechanism which also efficiently caused lethal damage to the hyphae which eventually inhibited the growth of *G. boninense*. As mentioned earlier, the mechanism of action for each anti-fungal compound was mainly based on their site of action (Ghannoum and Rice, 1999). The EtOAc extract of *T. virens* 159c might consist of anti-fungal compounds that caused the detachment of the hyphae which explains the difference in the modes of action of the EtOAc extract and Benlate®. Benlate, which is also known as benomyl is a wide spectrum fungicide that was designed to interfere with fungal cell division and could lead to disruption of microtubules (Hauptmann *et al*., 1985). Hexane extract from *T. virens* 7b reported by Angel *et al*. (2016) also showed different mode of damages to the hyphae of *G. boninense*. Therefore, it is important to characterise the *Trichoderma* strain to understand the role of antibiosis in suppressing the growth of *G. boninense*.

Based on the results, fractions 2 and 4 expressed highest anti-fungal activities towards *G. boninense* PER 71. These fractions constituted of acetamide, alcohol, lactones and free fatty acids that might be contributed to the suppression of the pathogen. Acetamide, N-(2-phenylethyl), was reported in the extract of *Inocybe geophylla* to have anti-fungal activity against the pathogenic fungi, *Fusarium oxysoprum* (Reinoso *et al*., 2013). Two fatty acids were identified; dodecanoic acid (fraction 2) and tetradecanoic acid (fraction 4). The higher percentage composition of tetradecanoic acid in fraction 4 of *T. virens* 159c might play a key role in the anti-fungal activities to give highest inhibition towards *G. boninense* PER 71. Fatty acids had been reported previously to have anti-fungal and anti-bacterial activities (Hilgren and Salverda 2000; Aneja *et al*., 2005; Pohl *et al*., 2011). They can insert themselves into lipid bi-layer of fungal membranes, physically disturb the membrane which caused the increased in fluidity that leads to conformational changes of membrane protein (Pohl *et al*., 2011). Other compounds identified from these active fractions was pyrrolo (1, 2a) pyrazine, 1,4-dione hexahydro-3-(2 methylpropyl), a pyrrolo ring derivative (methylpropyl). Similar pyrrolo compounds with methylpropyl derivatives from *Streptomyces* sp. were implicated in anti-fungal activities by Jog *et al*. (2014), where studies showed that *Streptomyces* strain that inhibits the growth of fungal pathogens produces this low molecular mass compound. However, compare to the GC-MSD profile of the *Streptomyces* strain in Jog *et al*. (2014), the level of detection for pyrrolo (1, 2a) pyrazine, 1,4-dione hexahydro-3-(2 methylpropyl) is higher compared to *T. virens* 159c. Another compound known as *dl*-mevalonic acid lactone was identified in fraction 4. The *dl*-mevalonic acid lactone was reported by Scopel *et al*. (2014) and significant inhibition to *Staphylococcus epidermidis* and interfere with its biofilm formation. However, *dl*-mevalonic acid lactone was never reported in *T. virens* and it was first discovered in this strain (*T. virens* 159c). This compound might contribute to the inhibition activity towards *G. boninense* PER 71 as it was detected at the active fraction of *T. virens* 159c. PEA was identified in fraction 2 of *T. virens* 159c, which was also identified in active band of *T. virens* 7b by Angel *et al.* (2016) and it was the first report of PEA to be secreted by *T. virens*. Band 5 of the hexane extract of *T. virens* 7b (Angel *et al*., 2016) and fraction 2 of the EtOAc extract of *T. virens* 159c efficiently suppressed the growth of *G. boninense* PER 71 probably due to the presence of PEA in both fractions. PEA showed strong inhibition against mycelial growth of fungal pathogens (Rouissi *et al*., 2013) and the anti-bacterial activity of PEA was also reported previously (Corre *et al*., 1990). However, there were three compounds

in common namely PEA, 3, 4-dimethylpent-2-en-1-ol and dodecanoic acid were compounds identified in the highly inhibitory fractions of extracts of both endophytic *Trichoderma*, namely the hexane extract of *T. virens* 7b (band 5) and the EtOAc extract of *T. virens* 159c (fraction 2). However, there was no report on the anti-fungal activity of 3, 4-dimethylpent-2-en-1-ol and its commercial product was not readily available for evaluation. As for dodecanoic acid, it was also found in the other fractions of *T. virens* extract that were not highly active. Hence, PEA was highlighted as unique discovery of *T. virens* because this compound was found only in highly inhibitory fractions from both endophytic *T. virens* 7b and 159c and was first reported to be produced by *T. virens*. The anti-fungal activity was proven with the commercial PEA which produced synthetically against *G. boninense* (Angel *et al*., 2016). These compounds identified from the active fractions might be responsible for the inhibition of *G. boninense* PER 71.

CONCLUSION

In this article, the study contributes significantly to the understanding of potential mechanisms involved in suppression of *G. boninense* by endophytic *T. virens* 159c. *T. virens* 159c showed significant and greater *Ganoderma* disease suppression in nursery and field trials conducted by Sundram *et al*. (2016) compared to *T. virens* 7b after application to the *Ganoderma* infected oil palm. Same species with two different extraction methods gave rise to different profiling of metabolites. Although both 7b and 159c are from *T. virens*, they differed in their performance when they were subjected to solvents, which prompted the investigation of using two different separation methods. An interesting discovery was made during the current study whereby PEA was discovered again in highly active fraction of *T. virens* 159c extract, accordance with the findings of Angel *et al*. (2016). In addition, *dl*-mevalonic acid lactone found in fraction 4 of *T. virens* 159c was first discovered in *T. virens*. The discovery was filed for patent. This study also shows that endophytic *T. virens* 159c have an excellent potential in the inhibition of *G. boninense* and the opportunity to introduce endophytes as biological control which serves as a better solution towards *Ganoderma* infection to the oil palm.

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