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Profiling of Metabolites Present in Ethyl Acetate Fractionation of *Trichoderma virens* 7b

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ABSTRACT

Endophytic Trichoderma virens *7b isolated from oil palm roots has been extensively studied to determine its potential against the oil palm pathogen,* Ganoderma. *The activities of its non-polar metabolites were previously reported to show the highest inhibition on* G. boninense *PER71 growth compared to other extracts. Nevertheless, it is also important to understand the activity of its semi-polar metabolites towards* G. boninense. *Therefore, this study aims to reveal the list of metabolites in* T. virens *7b extracted using ethyl acetate and the effect of extracts on* G. boninense *PER71. The ethyl acetate extract led to a significant decrease in the growth rate of* G. boninense *PER71 from 6.7 mm day^{-1} to 2.9 mm day^{-1}. This resulted in a percentage inhibition of radial growth (PIRG) of 59.63% ± 9.13. Coiling and clumping of the* G. boninense's *hyphae observed by scanning electron microscope subjected to ethyl acetate extract explain the growth inhibition. The metabolite profiles showed the presence of potential antifungal compounds which may contribute to the inhibition. These include succinimide and isolongifolene, 4,5,9,10-dehydro.*

ABSTRAK

Trichoderma virens *7b endofitik yang dipencil daripada akar pokok sawit telah dikaji untuk menentukan potensinya dalam menentang patogen sawit,* Ganoderma. *Aktiviti metabolit bukan polar yang dilaporkan sebelum ini telah menunjukkan perencatan pertumbuhan paling tinggi terhadap* G. boninense *PER71 berbanding ekstrak lain. Walau bagaimanapun, aktiviti metabolit semi-polar terhadap* G. boninense *adalah penting untuk dikaji. Oleh itu, kajian ini bertujuan untuk mengetahui profil metabolit dalam* T. virens *7b yang diekstrak menggunakan etil asetat dan kesan ekstrak terhadap* G. boninense *PER71. Ekstrak etil asetat berjaya mengurangkan kadar pertumbuhan* G. boninense *PER71 dari 6.7 mm hari^{-1} kepada 2.9 mm hari^{-1}. Hasilnya, peratus perencatan pertumbuhan* G. boninense *PER71 adalah sebanyak 59.63% ± 9.13. Cerapan mikroskop elektron imbasan (SEM) menunjukkan bahawa ekstrak etil asetat telah menyebabkan penggelungan dan pembentukan ketulan hifa* G. boninense *PER71, menjelaskan perencatan pertumbuhan* G. boninense *PER71. Profil metabolit menunjukkan kehadiran sebatian antifungal iaitu succinimide dan isolongifolene, 4,5,9,10-dehydro yang boleh menyumbang kepada perencatan* G. boninense *PER71.*

Keywords: biological control agent, *Trichoderma virens*, ethyl acetate, antifungal compounds.

INTRODUCTION

There is a potential use of naturally produced compounds to protect plants from attack by plant pathogens. Currently, microbes are seen as a greener alternative in reducing the possibility of crops succumbing to disease. The biological control processes such as plant defence mechanisms, mycoparasitism, competitions of nutrients, and antibiosis of *Trichoderma* are closely related with secondary metabolites (Hjeljord and Tronsmo, 1998.; Mukherjee *et al.*, 2012). A few classes of secondary metabolites, such as non-ribosomal peptides, terpenoids, pyrones, peptaboils, siderophores and polyketides have been reported to be secreted by different *Trichoderma*. These secondary metabolites are important for antibiosis (Howell *et al.*, 1993; Vinale *et al.*, 2006; El-Hasan *et al.*, 2009). Some metabolites released by potential biological control agents might cause damage to the pathogen which eventually inhibit their growth (Sharma and Sharma, 2008; Sundram *et al.*, 2011; Angel *et al.*, 2016, 2018).

In this current study, gas chromatography – mass spectrometry detector (GC-MSD) was used to identify the metabolites released by *T. virens* 7b. The technique is especially useful for identifying small molecular metabolites, such as small acids, hydroxyl acid, amino acids, alcohols and fatty acids

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(Fiehn, 2016). Metabolites identified from various *Trichoderma* using GC-MSD include compounds like D-limonene, 3-octanone, 2-hexanone, phenylethyl alcohol, 1-octene-3-ol and nonanal (Stoppacher *et al*., 2010; Siddiquee *et al*., 2012; Angel *et al*,. 2016, 2018). Fatty acids such as nonanoic acid, n-hexadecanoic acid, arachidic acid, octanoic acid had been reported in the profiling of *T. harzianum* (Siddiquee *et al*., 2012; Angel *et al*., 2016). They are known to exhibit antimicrobial and antifungal activities towards pathogens (Hilgren and Salverda, 2000; Pohl *et al*., 2011). In the case of *T. virens* 7b isolated from oil palm roots by Sundram (2013), an extensive study was conducted on the hexane extract (non-polar) and its effect against *G. boninense*. The hexane soluble fraction was identified to have the highest inhibition compared to the other extracts *i.e*. crude, ethyl acetate and butanol extracts (Angel *et al*., 2016). The active fraction constitutes compounds such as ketones, alcohols, aldehydes, lactones, sesquiterpenes, monoterpenes, sulphides and free fatty acids. The ethyl acetate soluble fraction of *T. virens* 7b was reported to possess antifungal activity but this particular fraction has not been extensively studied. Therefore, this study provides information on the metabolites released by *T. virens* 7b extracted using ethyl acetate and its activity towards *G. boninense*. The comparison between the ethyl acetate and hexane fraction of *T. virens* 7b is also discussed.

MATERIALS AND METHODS

Sample Preparation Method

The entire study was carried out using endophytic *T. virens* 7b, which was previously tested as a biological control agent against *G. boninense* (Sundram, 2013; Angel *et al*., 2016). *T. virens* 7b was grown on potato dextrose broth (PDB) in Erlenmeyer flasks at 28ºC on a shaker at 150 rpm. After 7 days, the culture filtrate was obtained by straining through the sterile muslin cloth. The metabolites were extracted as described previously by Angel *et al*. (2016) but using a semi-polar solvent, ethyl acetate as the extraction solvent. Ethyl acetate was evaporated using a rotary evaporator (Rotavapor® R-210/R-215 equipped with a controller V-850) to obtain dry extract.

Antifungal Assay and Microscopy Analysis

Antifungal assay was conducted using a modified well diffusion assay (Angel *et al*., 2016). Dried ethyl acetate extract of *T. virens* 7b was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg ml^{-1} and the antifungal assay was performed in 5 replicates for each test with three biological repeats. The growth of *G. boninense* PER71 was monitored on day 1, 3, 5, 7 and 8. Percentage inhibition of radial growth (PIRG) of *G. boninense* PER71 was calculated with the following equation:

$$PIRG\ (\%) = (R1 - R2) / R1 \times 100\%$$

R1: Negative control (DMSO only)

R2: Treated with ethyl acetate extract in DMSO

Based on the antifungal assay, the morphology of treated and untreated *G. boninense* PER71 mycelium was then assessed using a scanning electron microscope (SEM). The mycelium was fixed according to Angel *et al*. (2016).

Fractionation by Column Chromatography and Identification of Compounds using GC-MSD

The ethyl acetate extract was subjected to column chromatography using silica gel (230-400 mesh size, column 15 cm x 3.5 cm). The column was first eluted with 100% hexane, then further eluted with increasing percentage of dichloromethane, and lastly with 100% ethanol. A total of three main fractions collected based on band colour difference were collected and the solvents were removed using a rotary evaporator. Preliminary profiling of the metabolites from three fractions of *T. virens* 7b was carried out using Agilent Technologies 7890A GC-MSD based on the method conditions of Angel *et al*. (2016). The GC-MSD was equipped with a non-polar capillary column HP5 (30 m length × 0.25 mm i.d., 0.25 μm film thickness) and helium (99% purity) was used as the carrier gas at a flow rate of 2 ml min^{-1}. The injection volume was 1 μl. The oven temperature program was as follows: initial temperature at 50ºC (held for 4 min) and ramped at 5°C min^{-1} to 250°C. The injector temperature was maintained at 200°C in a split mode of 50:1, with a split flow of 20 ml min^{-1} at 1 min and a total flow of 54 ml min^{-1} with a scan range from m/z 35 to 500. The compounds were identified based on spectrum matching with reference compounds available in the National Institute of Standards and Technology (NIST) library at the Agilent GC-MS Mass Hunter Workstation.

RESULTS AND DISCUSSION

Antifungal activity by ethyl acetate extract. Based on the antifungal assay results published in Angel *et al*. (2018), ethyl acetate extract inhibited the growth of *G. boninense* PER71 with a PIRG of 59.63% ± 9.13. The inhibition by Benlate®, a proven commercial fungicide (positive control) was only 15.92% higher (Angel *et al*. 2016). The profile of growth decrease on the *G. boninense* PER71 can be observed in *Figure 1*. The standard growth rate of *G. boninense* PER71 of the negative control (DMSO only) was 6.7 mm day^{-1}. The reduction of the growth rate to 2.9 mm

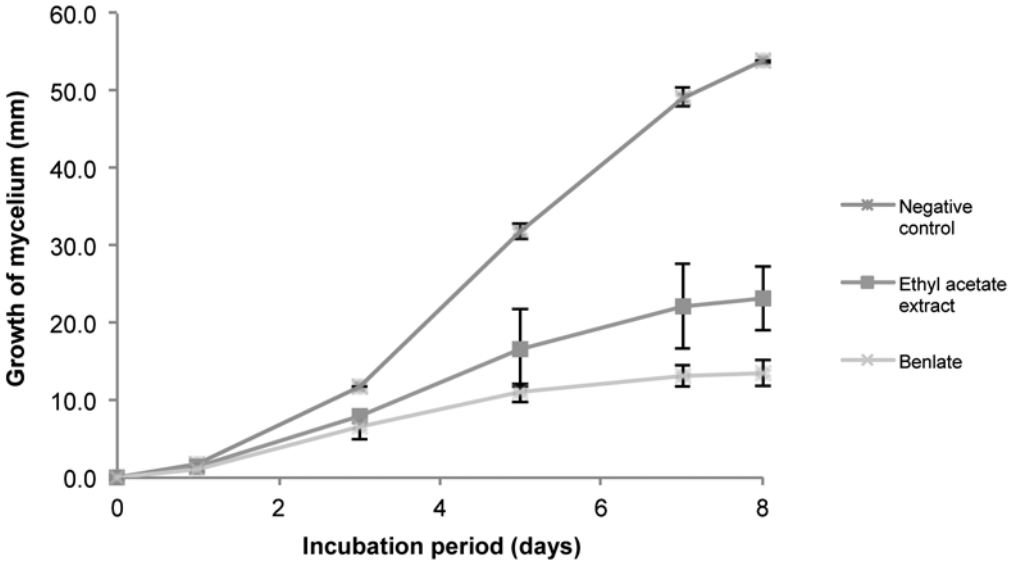

Figure 1. Progressive growth of G. boninense *PER71 for 8 days after treatment. Treatment: ethyl acetate extract; Negative control: DMSO only; Positive control: Benlate®.*

day^{-1} can be observed after introducing the ethyl acetate extract of *T. virens* 7b.

SEM showed distinct changes in the morphology of the hyphal structure of *G. boninense* PER71 after being treated with ethyl acetate extracts of *T. virens* 7b. The control plate (blank DMSO) showed a healthy and branched hyphal network of *G. boninense* PER71 with no abnormalities (*Figure 2a*). Coiling and clumping were observed in the *G. boninense's* hyphae subjected to ethyl acetate extract (*Figure 2b*). The damage on the cell walls was as severe as the hyphae treated with Benlate® (*Figure 2c*). Nevertheless, Angel *et al*. (2016) reported total collapse and severe damage of the hyphae system using the hexane extract. This could be explained by the presence of different antifungal compounds in different fractions, which employs different modes of action (Lorito *et al*., 1994; Sundram *et al.* 2011). The ethyl acetate extract was less destructive towards hyphae when compared to the hexane extract. This might also be due to the reduced amount of the responsible antifungal compounds in the particular fraction. These results explain the lower suppression of *G. boninense* PER71 growth by the ethyl acetate extract. The findings indicate that *T. virens* 7b produces both non-polar and semi-polar antifungal compounds. The semi-polar metabolites released by the isolates also played a role in inhibiting *Ganoderma* growth.

Metabolite identification. GC-MSD based metabolite profiling is a useful tool to gain an overview of the metabolites produced by plants or microorganisms. These metabolites include sugar alcohols, sugars, amino acids, fatty acids and organic acids (Obata *et al*., 2013). Metabolite profiling would help identify the reason behind the damage caused by the ethyl acetate extract of *T. virens* 7b towards *G. boninense* PER71 hyphae. Our results uncovered the preliminary profiles of the metabolites in the ethyl acetate fraction, which are listed in *Table 1*. The identification of metabolites was based on the percentage of matching spectra to the NIST library and only percentages of more than 70% were discussed in this article.

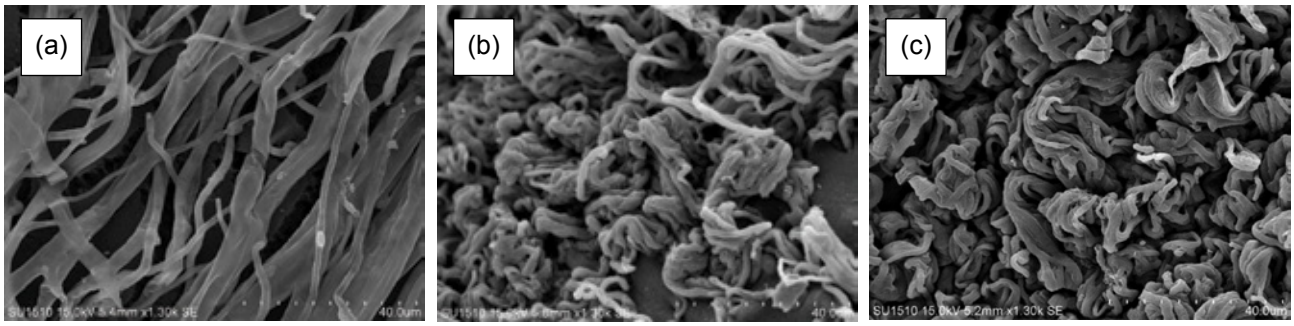

Figure 2. The characteristic of G. boninense *PER71 mycelium after treatment using scanning electron microscope (SEM) (40 µm); (a) DMSO only (Negative control); (b) ethyl acetate extract of* T. virens *7b; (c) Benlate® (Positive control).*

TABLE 1. METABOLITES IDENTIFIED FROM *T. virens* 7b ETHYL ACETATE EXTRACT

	Compounds	Retention time (min)	Matching of spectra (%)
Fraction 1			
1	1,2,2,3- tetramethylcyclopent-3-enol	8.334	41.5
2	**Succinimide**	**9.077**	**94.5***
3	**Isolongifolene,4,5,9,10-dehydro**	**11.907**	**74.8***
4	Heptadecanoic acid, methyl ester	12.062	58.4
Fraction 2			
1	Propanedioic acid, propyl-	5.317	63.30
2	Cycloserine	6.831	42.50
3	1-2,4-Bis(trimethylsiloxy)phenylpropan-1-one	7.633	56.90
4	**S-methyl methanethiosulphonate**	**8.451**	**96.70***
5	Heptanoic acid	8.505	44.20
6	Nonanal	8.874	55.40
7	3,4-Epoxytetrahydrothiophene-1,1-dioxide	9.526	57.70
8	Isolongifolene,4,5,9,10-dehydro	11.885	65.50
9	Acetamide, N-(2-phenylethyl)-	12.078	64.70
Fraction 3			
1	Propanedioic acid, propyl-	7.275	44.8
2	**1-2,4-Bis(trimethylsilyloxy)phenylpropan-1-one**	**7.638**	**70.3***
3	1-propanone,2-bromo-1-phenyl	8.478	44.8
4	Propanamide,2-hydroxy-	9.232	47.1
5	3,4-epoxytetrahydrothiophene-1,1-dioxide	9.532	40.8
6	Nonanoic acid	10.259	44.4

Note: * Compounds with high spectral similarity to compounds in the NIST library.

Succinimide was found in the first fraction of the ethyl acetate extracts with 94.5% match to the compounds in the NIST library. They are known to be an important class of substrates for biological, pharmacological and chemical applications (Shetgiri and Nayak, 2005). Succinimide is part of many active molecules that possesses antibacterial and antifungal effects. This compound was tested against several microorganisms, including *Staphylococcus aureus*, *Streptococcus pyogene*, *Candida albicans* and *Escherichia coli*, resulting in growth inhibition (Al-Azzawi and Hassan, 2014; Sortino *et al*., 2013).

Isolongifolene,4,5,9,10-dehydro was also identified in the extract. It is a type of sesquiterpene that is associated with potential antifungal activities (Jagatheesh *et al*., 2013). Isolongifolene,4,5,9,10-dehydro was previously reported in *Cinnamomum zeylanicum* (Cinnamon bark). The metabolite was found in the methanolic extract and was able to inhibit pathogenic microbes such as *Aspergillus flavus*, *E. coli* and *Klebsiella pneumonia*. Nevertheless, individual activity of isolongifolene,4,5,9,10-dehydro has not been reported (Hameed *et al*., 2016).

S-methyl methanethiosulphonate was a compound found in fraction 2. It is a temporary enzyme-sulfhydryl blocking agent that is known to be a potent bio-antimutagen in *E. coli* (Nakamura *et al.*, 1997). This compound was previously reported to be produced by *Burkholderia ambifaria*. High concentrations of S-methyl methanethiosulphonate together with other compounds inhibited growth of phytopathogenic fungi such as *Fusarium culmorum*, *F. oxysporum*, *Colletotrichm gloesporiodes* and *Sclerotum rolffsi* (Groenhagen *et al*., 2013). As for 1-2,4-Bis(trimethylsilyloxy)phenylpropan-1-one identified in fraction 3, to the best of our knowledge, its activity in plants has not been reported in the literature.

It is important to note that the present classification of the metabolites is preliminary, and require further optimisation of the GC-MSD

method or verification using other techniques, such as Nuclear Magnetic Resonance to confirm the identified compounds. The list of metabolites provides a possible indication of compounds secreted by *T. virens* 7b. More research efforts are required to isolate, characterise, evaluate and verify these compounds in future research. It is necessary to validate their identity and antifungal importance towards pathogens before making any conclusion on the presence and role of the profiled compounds.

CONCLUSION

In this preliminary study, the research shows the activity of ethyl acetate extract of *T. virens* 7b towards *G. boninense* PER71 and the composition of the metabolites in the different fractions. The identification of metabolites provides a greater understanding of the mode of action of the compounds in the ethyl acetate extract. The metabolites can disrupt and damage the hyphae of *G. boninense* PER71, thereby slowing its growth. The ethyl acetate fraction was further fractionated by column chromatography to identify metabolites by GC-MSD, giving a list of potential compounds for further study. Fractions collected from the ethyl acetate extract were not tested in detail for their antifungal activities. The findings in this paper are to highlight the presence of various metabolites extracted using ethyl acetate that will serve as a guide for future study of this fraction. It also provides additional information towards the understanding and potential application of *Trichoderma* as a biofungicide to complement the previously reported findings of hexane extract.

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