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Volatile organic compounds profiles emitted by *Cochliobolus miyabeanus*, a causal agent of brown spot disease of rice

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ABSTRACT

Aims: Brown spot disease is among the important crop diseases of rice caused by the infection of a pathogenic fungus, *Cochliobolus miyabeanus* that results in yield losses. Nowadays, limited studies on volatile organic compounds (VOCs) have been carried out using pathogenic fungal isolate. Hence, this study was conducted to identify VOCs produced by *C. miyabeanus* wild-type isolate, WK1C, a causal agent of brown spot disease using gas chromatography-mass spectrometry (GC-MS).
Methodology and results: Fungal isolate WK1C was cultured on potato dextrose agar (PDA) and in potato dextrose broth (PDB) for extraction. The extracts were analysed using GC-MS and the profiles of VOCs were obtained. *Cochliobolus miyabeanus* WK1C isolate showed a significant presence of various types of organic compound including ester, alcohol, phenol, alkane, alkene, ketone, carboxylic acid, amide and aldehyde.
Conclusion, significance and impact of Study: This study important for a preliminary assessment of VOCs profiles of *C. miyabeanus*, a causal agent of brown spot disease. In order to identify the compounds contribute to pathogenicity, further study can be conducted to identify the virulence factor of brown spot disease using different approaches.

Keywords: Cochliobolus miyabeanus, fungi, volatile organic compounds (VOCs), metabolite identification, gas chromatography- mass spectrometry (GC-MS)

INTRODUCTION

Cochliobolus miyabeanus (*Bipolaris oryzae*) is one of the species under the order of Pleosporales that can cause brown spot disease, which is widely known as one of the important crop yield diseases of rice (*Oryza sativus* L.) (Savary *et al.*, 2012). The rice brown spot disease caused by *C. miyabeanus* is a widespread crop disease especially in South and South-East Asian countries and has been reported as the highest crop yield loss (Savary *et al.*, 2000). This situation can cause a huge impact on the economic since rice is a staple food for Asian countries and commonly consumed by the populations.

Cochliobolus species produces certain products or compounds through metabolic process including primary and secondary metabolisms. In fungi, primary metabolites are often defined as essential compounds that play an important role in growth of the fungi. The primary metabolism compounds include carbohydrates, proteins, nucleic acid and lipids (Kavanagh, 2011). In contrast to primary metabolites, secondary metabolites are not essential for growth or development, but known to aid fungi to successfully compete with other organisms in its natural habitat. Secondary metabolites can be classified into distinct groups or classes such as polyketides, fatty acids, phenylpropanoids, aromatic amino acids, terpenoids, alkaloids and non-ribosomal peptides (Dewick, 2009).

Many fungi include *Cochliobolus* species are known to produce volatile organic compounds (VOCs) responsible in ecological and physiological roles. VOCs comprised the mixture of gas-phase, carbon-based compounds that can be easily evaporated into the environment due to their small size. VOCs are commonly produced via industrial activities, but most of them are the products from diverse metabolic processes. According to Korpi *et al*. (2009), most of fungal VOCs emissions were derived from biological metabolic pathways including primary and secondary metabolisms. Plant VOCs and bacterial VOCs have been extensively studied compared to fungal VOCs (Schulz and Dickschat, 2007; Piechulla and Degenhardt, 2014; Kanchiswamy *et al.*, 2015). Approximately, 250 natural products of VOCs have been reported to produce by fungi that comprised simple hydrocarbons, aldehydes, ketones, alcohols, phenols, benzene derivatives and other group compounds (Chiron and Michelot, 2005;

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Korpi *et al.*, 2009; Ortiz-Castro *et al.*, 2009). The production of VOCs emitted by fungi varies within species, growth conditions and environment factors such as temperature, moisture level, nutrients and pH (Morath *et al.*, 2012).

The production of VOCs from fungal secondary metabolism is usually associated with the formation of spore and mycotoxin activity, which capable of causing diseases to host plants. At present, there are limited literatures or studies about the toxigenic VOCs emitted by fungi especially from genus *Cochliobolus* (Bennet and Inamdar, 2015) as most of published studies on fungal VOCs were focused on economic importance. Therefore, the main aim of this study is to identify VOCs produced by *C. miyabeanus* WK1C, a causal agent of brown spot disease of rice using GC-MS. Findings from this work are the key for future study on virulence factors involved in pathogenesis of brown spot disease of rice.

MATERIALS AND METHODS

Fungal isolates

Cochliobolus miyabeanus WK1C was obtained from Mycology Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia (UPM). The fungal isolate was cultured and grown on potato dextrose agar (PDA) plates at room temperature for seven days. Three pieces of mycelia plugs from the fungal culture were inoculated in conical flask (250 mL) containing 50 mL of potato dextrose broth (PDB) in three replicates (Siddiquee *et al.*, 2012). The flasks were then incubated in shaking incubator (150 rpm) at room temperature in darkness for three days.

Extraction of fungal compounds

According to the method described by De Bruyne *et al.* (2016) with slight modification, the inoculated PDB culture was blended using blender (Waring Commercial Blender) after three days of incubation to homogenise the mycelia. About 1 mL of homogenised mycelial suspension (blended mycelia) was transferred using micropipette into conical flask containing 30 mL of fresh PDB. The flask was then incubated in shaking incubator (150 rpm) at room temperature in darkness for another seven days. The culture was harvested after that where the mycelia were separated through two layers of sterile cheesecloth followed by one layer of Whatman No.1 filter paper to remove any solids. Syringe filter with pore size of 0.22 µm was used to sterilise the filtrate and remove any conidia present. Every 4 mL of the crude filtrate collected was added with 1.8 g of magnesium sulfate ($MgSO_4$) that acts as a drying agent.

The crude filtrate was then extracted with 10 mL of organic solvent, which is a mixture of ethyl acetate: acetonitrile (1:1, v/v). The filtrate was vigorously shaken on orbital shaker for 30 min and the filtrate was separated using separatory funnel to collect the organic upper phase. The organic upper phase was collected into the conical flask while extracted aliquot was air dried under fume chamber for several days before GC-MS analysis.

Gas Chromatography-Mass spectrometry (GC-MS) analysis

Separations of VOCs were determined using GC-MS, (QP2010 Plus SHIMADZU). GC-MS analyses were done with ionisation energy of 70 eV. Putative identification of metabolites was performed using non-polar column as stationary phase. For the non-polar column, ZB-5MS 30 m × 0.25 mm ID × 0.25 µm film thickness of capillary column was used with the volume of injected sample of 1 µL.

The oven programme had an initial temperature of 60 °C for 1 min, then a 5 °C/min run to 300 °C with a final hold at 300 °C (5 min). The injector temperature was kept at 300 °C (split less) and detector temperature was at 320 °C. In GC-MS, typical mobile phase involves inert gases such as helium, nitrogen and hydrogen. For this analysis, helium was used as the carrier gas at a linear flow rate of 1 mL/min and linear velocity of 36.5 cm/sec, which is the optimal linear velocity that gives the best efficiency, while the MS detector was operated at 194 °C. Mass spectral scan range was set at 35 to 450 (m/z) at a scan rate of 0.50 scan/sec.

Identification of fungal VOCs

For identification of VOCs, the interpretation on mass-spectrum GC-MS was conducted using database of Natural Institute Standard and Technology (NIST) with more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight and molecular formula of the extract samples were ascertained.

RESULTS AND DISCUSSION

Three replicates for *C. miyabeanus* WK1C culture were analysed using GC-MS and the presence of VOCs was determined. Based on the obtained result, there were several organic compounds from various groups detected from the cultures such as the group of ester, alcohol, phenol, alkane, alkene, ketone, carboxylic acid, amide, and aldehyde. Eighty compounds were produced by the culture as listed in Table 1. Details of the compounds identified can be observed from the chromatograms as shown in Figure 1. The culture produced significant and high amount of ester [2-ethoxyethyl acetate and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester], phenol [phenol, 2,4-bis (1-phenylethyl)] and alkane (heneicosane).

Gas Chromatography-Mass Spectrometry (GC-MS) technique, which is a chromatography technique with mass spectrometer as a detector, was utilised to identify the production of VOCs by *C. miyabeanus* isolate. identification of the compounds can be commonly achieved by comparing mass spectra with NIST database

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Table 1: List of volatiles compounds produced by isolate WK1C detected using GC-MS.

No.	RT	Area %	Name of compound	Group of compound
1.	5.367	0.47	Methyl N-hydroxybenzenecarboximidoate	Ester
2.	5.530	2.78	2-Ethoxyethyl acetate	Ester
3.	6.604	1.78	3-Hydroxy-4-methoxybenzaldehyde, tert-butyldimethylsily ether	Ether
4.	7.533	4.08	Phenol	Phenol
5.	9.479	0.48	2-Pyrrolidinone, 1-methyl-	Ketone
6.	9.668	0.97	Benzoic acid	Carboxylic acid
7.	10.235	0.61	3-methyl-phenol	Phenol
8.	10.821	1.38	1,2-Benzisothiazole	Ketone
9.	10.897	0.81	2,5-bis[(trimethylsilyl)oxy] benzaldehyde	Aldehyde
10.	11.401	0.89	Phenylethyl Alcohol	Alcohol
11.	12.393	0.74	2,4-dimethyl-phenol	Phenol
12.	13.566	0.52	1-(3-methylphenyl)-ethanone	Ketone
13.	16.682	4.06	2-Benzothiazolinone	Ketone
14.	17.147	2.95	2-(1-phenylethyl)-phenol	Phenol
15.	17.461	0.81	Tetradecanoic acid	Carboxylic acid
16.	18.072	0.71	1,6-dimethyl-4-(1-methylethyl)-naphtelene	Alkane
17.	18.604	2.06	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	Ester
18.	19.011	0.51	Chloroxylenol	Alcohol
19.	19.366	0.81	10-(2-hexylcyclopropyl) decanoic acid	Carboxylic acid
20.	19.567	4.72	1-(+)-Ascorbic acid 2,6-dihexadecanoate	Ester
21.	19.901	1.36	2,2-Dimethyl-4-octenal	Aldehyde
22.	19.963	0.99	Tetracosane	Alkane
23.	20.335	0.55	4b,8-Dimethyl-2-isopropylphenanthrene, 4b,5,6,7,8,8a,9,10-octahydro	Alkene
24.	20.934	1.65	2,2-Dimethyl-4-octenal	Aldehyde
25.	21.014	1.58	2(3H)-Furanone, 5-dodecyldihydro	Ketone
26.	21.223	2.15	9,12-Octadecadienoic acid (Z,Z)	Carboxylic acid
27.	21.297	18.35	9-Octadecanoic acid, (E)	Carboxylic acid
28.	21.315	0.71	5-(3-Phenyl-propenyl)-dihydrofuran-2-one	Ketone
29.	21.479	1.38	Octadecanoic acid	Carboxylic acid
30.	21.829	0.65	Pentacosane	Alkane
31.	22.191	0.84	6-isopropenyl-1,2,3,4-tetramethyl-1,4-Cyclohexadiene,	Alkene
32.	22.320	1.66	N,N-dimethyl octanamide,	Amide
33.	22.645	1.26	9,12-Octadecadien-1-ol, (Z,Z)	Alcohol
34.	22.700	1.30	Dotriacontane	Alkane
35.	22.898	0.83	cis-9-Hexadecenal	Aldehyde
36.	22.953	0.50	1,2,3-trimethyl-, ethyl ester,(.+-.)- 2-cyclopentene-1-carboxylic acid,	Ester
37.	23.307	0.87	Abiet-8-en-18-oic acid	Carboxylic acid
38.	23.842	3.36	N,N-dimethyl-9-Octadecenamide,	Amide
39.	24.018	6.83	1-Phenanthrenecarboxylic acid	Carboxylic acid
40.	24.130	0.53	Eicosanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	Ester
41.	24.298	4.51	Phenol, 2,4-bis(1-phenylethyl)	Phenol
42.	24.817	1.84	Bis[2-(3,5-di-tert-butylbenoyloxy)-1-naphthyl]methane	Alkane
43.	25.115	0.96	Hexatriacontane	Alkane
44.	25.991	0.52	2(3H)-Benzothiazolone	Ketone
45.	26.358	1.76	Propennitrile, 2-(benzoaxazol-2-yl)-3-hydroxy	Nitrile
46.	26.590	2.02	Oxazolidin-2-one, 3-tert-butyl-5-spiro-cyclohexane-4-hydroxy-4-methyl	Ketone
47.	26.780	0.87	6.beta.Bicyclo[4.3.0]nonane, 5.beta.-iodomethyl-1.beta.-isopropenyl-4.alpha.,5.alpha.-dimethyl-	Alkane
48.	27.015	4.65	Azulene, 1,4-dimethyl-7-(1-methylethyl)	Alkene
49.	27.086	0.95	1,2-Diphenylcyclopropane	Alkane
50.	27.414	0.73	1H-Inden-1-ol, 2,4,5,6,7,7a-hexahydro-4,4,7a-trimethyl-	Alcohol
51.	28.258	4.57	Cyclononasiloxane, octadecamethyl	Alkane
52.	28.493	3.60	Cannabinol, trifluoroacetate	Ester

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Table 1: Continued.

53.	28.774	0.80	Heptadecane	Alkane
54.	29.142	0.98	Tetradecanal	Aldehyde
55.	29.517	0.66	Sulfamerazine	Amide
56.	29.546	0.78	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Ketone
57.	30.017	0.55	Phthalic acid, diisobutyl ester	Ester
58.	30.832	1.53	Nonadecane	Alkane
59.	31.266	3.49	Heptasiloxane, hexadecamethyl	Alkane
60.	31.553	2.12	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1'2'-d]pyrazine	Pyrazine
61.	31.921	4.02	Dibutyl phthalate	Ester
62.	31.994	0.66	n-Hexadecanoic acid	Carboxylic acid
63.	32.683	3.08	Hexadecanoic acid, ethyl ester	Ester
64.	32.809	0.96	Heneicosane	Alkane
65.	35.328	1.22	Oleic acid	Carboxylic acid
66.	35.428	0.46	Cis-9-Hexadecanal	Aldehyde
67.	36.379	1.19	Octadecanoic acid, ethyl ester	Ester
68.	37.379	0.74	N,N-Dimethyldodecanamide	Amide
69.	38.388	0.57	7-(2,6-Dimethyl-hepta-1,5-dienyl)-3,8,8-trimethyl-bicyclo[4.2.0]oct-2-ene	Alkene
70.	40.333	1.61	9-Octadecenamide, N,N-dimethyl	Amide
71.	40.503	3.52	(2-Phenyl-3-[(phenylsulfinyl)methyl]cyclopropyl) benzene	Benzene
72.	41.076	13.22	Phenol, 2,4-bis(1-phenylethyl)	Phenol
73.	41.467	0.82	Eicosane	Alkane
74.	41.914	3.34	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Ester
75.	44.477	0.79	Tetratetracontane	Alkane
76.	47.825	0.53	Benzene, 1,3,5-triphenyl-	Alkene
77.	48.782	10.08	4,5-2H-Oxazole-5-one, 4-[3,5-di-t-butyl-4-methoxyphenyl]methylene-2-phenyl	Ketone
78.	49.014	10.07	5,5',8,8',-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone	Ketone
79.	49.333	0.65	1,3,4,5-Tetraphenyl-4-imidazoline-2-thione	Ketone
80.	51.858	0.45	1.alpha.,2.alpha.,3.alpha.,4.beta. 1,2,3,4-tetrahydrobenz[a]pyrene-2,3,4-triol-1-amine[a]pyrene, tribenzoate	Ester

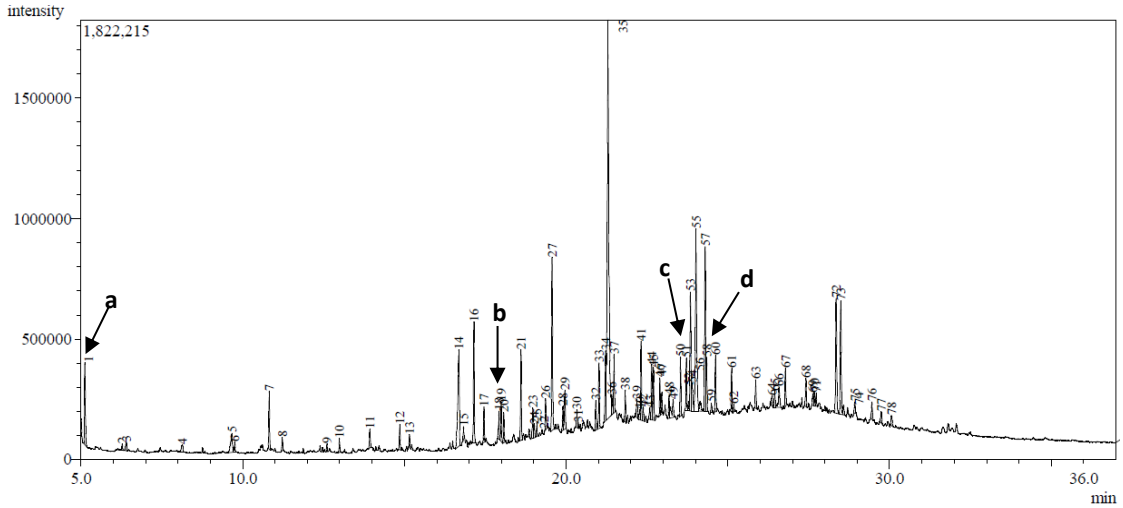

Figure 1: Chromatogram of VOCs identified from *C. miyabeanus* WK1C using GC-MS. (a) 2-Ethoxyethyl acetate; (b) Heneicosane; (c) Phenol, 2,4-bis (1-phenylethyl); (d) 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester.

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(Oprean *et al*., 2001; Jelen, 2003) or by standard comparison. In this study, spectral library comparison was applied instead of using standard comparison since no specific compounds of VOCs were to be analysed by GC-MS; thus, all the VOCs produced by the isolate were screened. In the GC-MS, prior to perform the analysis, the condition of the instrument was set up according to the suitability of the sample subjected and objective of this study. In this case, non-polar column was used as stationary phase. Hence, the polar compounds were eluted out first, whereas less polar compounds bound to the stationary phase were eluted out later due to the strong interaction between the compound and the stationary phase. The aim of this study is to screen all compounds produced by different isolates without considering the polarity characteristic.

Even though some VOCs were able to identify using GC-MS, there was no report or supporting evidence available regarding the compounds identified from this study with fungal virulence association. Moreover, the studies about VOCs produced by plant pathogenic fungi especially from species *Cochliobolus* species were limited and most studies regarding VOCs were involved in endophytic fungi for ecological importance such as that by Siddiquee *et al.* (2012), which reported the VOCs produced by *Trichoderma harzianum* as biocontrol fungus. Therefore, this is the first report on VOCs profiles produced by the studied isolate. Further study must be carried out to confirm the role of the compounds emitted to the rice as a plant host during pathogenesis of brown spot disease.

In this study, GC-MS analysis was only allowed for detecting VOCs at certain concentrations. According to Chiang *et al.* (2011), in laboratory condition, cultivated wild type normally produces very low amount of secondary metabolites as many genes responsible for production of the metabolites are remained silent under this condition. Optimising the environment factors such as temperature and humidity helps to increase the production of metabolite compounds by the fungi (Sorensen *et al.*, 2013), thus in the future study it is recommended that increasing the concentration of the compounds, thus they can be detected by GC-MS analysis.

CONCLUSION

Several organic compounds from various groups such as ester, alcohol, phenol, alkane, alkene, ketone, carboxylic acid, amide and aldehyde were successfully detected in extract of *C. miyabeanus* wild-type isolate, WK1C, a causal agent of brown spot disease.

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