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Optimization of Metabolite Extraction Protocols for Untargeted Metabolite Profiling of Mycoparasitic *Scytalidium parasiticum* using LC-TOF-MS

(Pengoptimuman Protokol Pengekstrakan Metabolit Tak Bertumpu bagi Profil Metabolit daripada Mikoparasit *Scytalidium parasiticum* Menggunakan LC-TOF-MS)

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ABSTRACT

Basal stem rot disease of oil palm caused by Ganoderma boninense *is one of the most devastating diseases in oil palm plantation resulting in low yield, loss of palm stands and shorter replanting cycle. To-date, there is no effective treatment for* Ganoderma *infected palms. Control measures, either chemical or cultural approaches, show varying degrees of effectiveness. The application of biological control agents which is environmental-friendly could be an attractive solution to overcome the problem. Earlier, we had isolated a mycoparasite,* Scytalidium parasiticum, *from the basidiomata of* Ganoderma boninense. In vitro *assay and nursery experiment showed that this fungus could suppress* Ganoderma *infection and reduce disease severity. However, metabolites which might contribute to the antagonistic or mycoparasitic effect remain unknown. In the current study, optimization of fungal sample processing, extraction, and analytical procedures were conducted to obtain metabolites from the maize substrate colonized by mycoparasitic ascomycetous* Scytalidium parasiticum. *This technique capable of producing sexual spores in sac-like organs. Untargeted metabolomics profiling was carried out by using Liquid Chromatography Time of Flight Mass Spectrometry (LC-ToF-MS). We found that* S. parasiticum *in both liquid- and solid-state cultivation gave higher metabolite when extracted with 60% methanol with 1% formic acid in combination with homogenisation methods such as ultrasonication and grinding. The findings from this study are useful for optimisation of metabolite extraction from other fungi-*Ganoderma*-plant interactions.*

Keywords: Biocontrol agent; Ganoderma boninense*; LC-ToF-MS; metabolomics; oil palm*

ABSTRAK

Penyakit busuk pangkal batang bagi pokok kelapa sawit yang disebabkan oleh Ganoderma boninense *adalah salah satu penyakit yang paling serius di ladang sawit yang mengakibatkan hasil yang rendah, kekurangan pokok sawit yang produktif dan kitaran penanaman semula yang lebih singkat. Sehingga kini, tiada rawatan berkesan untuk pokok sawit yang dijangkiti* Ganoderma. *Langkah-langkah kawalan, sama ada pendekatan kimia dan teknik pengkulturan, menunjukkan tahap keberkesanan yang berlainan. Penggunaan agen kawalan biologi yang mesra alam boleh menjadi langkah alternatif yang terbaik dalam menangani masalah ini.* Scytalidium parasiticum *telah dipencilkan daripada basiodamata* G. boninense. *Kajian* in vitro *dan nurseri telah menunjukkan bahawa kulat ini boleh merencatkan jangkitan* Ganoderma *dan mengurangkan kesan yang lebih parah terhadap penyakit ini. Walau bagaimanapun, metabolit yang mungkin menyumbang kepada kesan antagonistik atau mikoparasit masih tidak diketahui. Dalam kajian ini, pengoptimuman pemprosesan sampel, pengekstrakan dan analisis dilakukan untuk mendapatkan metabolit daripada mikoparasit askomise* S. parasiticum *yang dikultur dengan substrat jagung. Teknik ini mampu menghasilkan spora seksual dalam organ seperti sac. Pemprofilan metabolomik tidak bertumpu dilakukan dengan Kromatografi Cecair Ion Terbang Spektrometri Jisim (LC-ToF-MS). Kami mendapati bahawa* S. parasiticum *yang dikultur dalam media cecair dan dalam keadaan pepejal memberikan jumlah metabolit lebih tinggi apabila diekstrak dengan 60% metanol dan 1% asid formik dengan kombinasi kaedah penghomogen seperti ultrasonikasi dan pengisaran. Keputusan kajian ini adalah berguna untuk mengoptimumkan penghasilan metabolit daripada ekstrak interaksi kulat-*Ganoderma*-sawit.*

Kata kunci: Agen biokawalan; Ganoderma boninense*; kelapa sawit; LC-ToF-MS; metabolomik*

INTRODUCTION

Basidiomycetous *Ganoderma boninense* Pat., the main causal agent of white rot disease or also known as basal stem rot (BSR) and upper stem rot (USR) diseases in oil palm (*Elaeis guineensis* Jacq.), together with a few other *Ganoderma* species, were reported to harbour a series of fungicolous fungi (Agustini et al. 2014; Chaverri et al. 2015; Gams et al. 2004; Helfer 1991; Kang et al. 2011; Põldmaa et al. 1999; Rogerson & Samuels 1993). In a few recent studies related to fungiculous fungi associated with *G. boninense*, *Cladobotryum semicirculare* G.R.W. Arnold, R. Kirschner & Chee J. Chen initially isolated from *G.*

tsugae Murrill (Kirschner et al. 2007), was also reported from *G. boninense* (Marzuki et al. 2015); and Scytalidium *parasiticum* Y-Kheng Goh, Goh, Y.K. Goh, K.J. Goh was isolated from *G. boninense* (Goh et al. 2015).

Secondary metabolites, namely yellow pigments from *Scytalidium ganodermophthorum* suppressed the growth of *G. lucidum* and were postulated to assist in *Ganoderma* host parasitism as well (Kang et al. 2010; Oh et al. 1998). Kang et al. (2014) attempted to extract the antifungal metabolites from *S. ganodermophthorum* with methanol and ethyl acetate; both methanol and ethyl acetate extracts illustrated growth inhibition in *Phytophothora capsici* Leonian and other fungal pathogens at the concentration of 100 ppm or higher. *In vitro Ganoderma* pathogenicity tests showed the capability of *S. ganodermophthorum* in reducing the survival of *G. lingzhi* only, whereas, the newly described potential necrotrophic mycoparasitic *S. parasiticum* was able to reduce the survival of *G. boninense* and other tested *Ganoderma* species (Goh et al. 2016). Necrotrophic mycoparasites normally kill the host cells prior to occupying them and followed by extracting nutrients from the dead cells (Boosalis 1964). In a preliminary experiment conducted in 2014, *S. parasiticum*-maize methanol extracts were found to be inhibitory to the growth of *G. boninense*, on contrary; there was no inhibition in when *S. parasiticum*-maize ethyl acetate was used (unpublished data). The major functions and identity of dark yellowish and green fluorescent pigments produced by *S. parasiticum* inoculated on maize or during the interactions with *Ganoderma* were still unknown. To the best of our knowledge, the information related to the metabolomics study on fungicolous or mycoparasitic fungi in close association with *Ganoderma boninense* is limited especially since *Scytalidium parasiticum* is a newly identified and described parasite of *G. boninense*. In this study, a series of metabolite extraction protocols for *S. parasiticum* were performed and evaluated prior to LC-ToF-MS analyses. These protocols were based on: Choice of extraction solvents - 60% methanol amended with 1% formic acid, 100% methanol, 100% acetonitrile, and water only; state (liquid/solid state) of fungus cultivation; and homogenisation methods (grinding vs non-grinding and ultrasonication vs non-sonication). Optimized protocols will be useful for future metabolomics studies on bitrophic *Scytalidium-Ganoderma* or *Scytalidium*-plant, and tritrophic *Scytalidium-Ganoderma*-plant interactions, as well as screening for potential antifungal compounds to manage *G. boninense*.

Materials and Methods

Chemicals

Methanol and acetonitrile were purchased from Merck, Germany. Formic acid and caffeic acid for LC-ToF-MS analyses were purchased from Sigma-Aldrich, USA. All solvents used were MS grade.

Fungal isolate and culture conditions

Ascomycetous, potentially necrotrophic mycoparasitic *Scytalidium parasiticum* AAX0113 was maintained on malt extract agar (MEA; Difco) and incubated at 24°C in the dark for 7 days prior to transferring the mycelial plugs (1 cm^2 in diameter) onto sterilized maize (dry kernels) and incubated for an additional 14 days. Solid-state cultivation: Fourteen-day-old *S. parasiticum*-inoculated sterilized maize was prepared for metabolites extraction in most of the experiments, unless otherwise specified. Liquid-state cultivation: Maize was blended into powder using a Waring® laboratory blender and approximately 75 g of maize powder was added into 500 mL of double distilled water (ddH_2O) to produce maize extract broth (MaEB). Maize sterilization was carried out according to Goh et al. (2016) prior to inoculating with *S. parasiticum*.

Fungal metabolites extraction and optimization

Fungal metabolites extraction with samples from solid-state cultivation: Twenty mL 60% cold methanol supplemented with 1% formic acid was added into 20 g maize inoculated with *Scytalidium parasiticum* or 20 g un-inoculated maize and the mixtures were vortexed vigorously for 30 s, followed by frozen in liquid nitrogen for 5 min. The samples were then thawed on ice for 10 min. Methanol extracts were transferred to new 1.5 mL Eppendorf tubes and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then transferred to new cold tube and the precipitated pellet was re-extracted with 60% cold methanol amended with 1% formic acid. The extracts were then vortexed for 30 s and followed by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatants from first and second extractions were combined and concentrated for 4 to 6 h using vacuum concentrator (Eppendorf Concentrator plus). The samples were stored at -80°C prior to metabolomics analysis.

Fungal metabolites extraction with samples from liquid-state cultivation: MaEB inoculated with *S. parasiticum* or the non-inoculated MaEB were incubated at 24°C on a rotary shaker at 150 rpm for 2 weeks. Both the inoculated and non-inoculated MaEB were centrifuged at 7000 rpm for 15 min at 4°C. The extracts were filter-sterilized with 0.45 µm filter (Whatman). Three hundred µL of the extracts were transferred to 900 µL 60% cold methanol with 1% formic acid and the mixtures were vortexed vigorously for 3 s. The samples were then concentrated for 4 to 6 h using vacuum concentrator and stored at -80°C prior to metabolomics analysis.

Two other solvents, namely 100% acetonitrile and water were selected for fungal metabolites extraction with samples from both solid- and liquid-state cultivations. These two solvents were compared to 60% methanol supplemented with 1% formic acid.

OPTIMIZATION 1: WITH AND WITHOUT VACUUM CONCENTRATION

During fungal metabolites extraction, once the supernatants from the first and second extractions were combined, the combined supernatants were concentrated in vacuum concentrator for 4 to 6 h. The supernatants without vacuum concentration were used as the control. The effects of vacuum concentration on the extracted supernatants were evaluated. There was no difference in the number of metabolite features (21 peaks) recorded for both treatments - with and without concentration (Data not shown).

OPTIMIZATION 2: GRINDING VS NON-GRINDING

Scytalidium parasiticum-inoculated and non-inoculated maize were subjected to grinding Waring® blender prior to solvent extraction. The non-grounded samples were used for comparison as well.

OPTIMIZATION 3: SONICATION VS NON-SONICATION

Scytalidium parasiticum-inoculated and non-inoculated maize were subjected to sonication in an ice bath with a sonic dismembrator (Fisher Scientific, FB120) fitted with a Model CL-18 probe at 65% power and 30% amplitude with 15 s pulses. The sonication process was then repeated 4 times after a one-minute pause in between each cycle. The non-sonicated samples were used as the control.

LC-TOF-MS ANALYSES

Fungal metabolites extracted from *S. parasiticum*-inoculated and non-inoculated MaEB (liquid-state cultivation) were selected for optimizing the LC-MS protocols, namely flow rates, gradients, oven temperatures and columns. Mobile phases contained water + 0.1% formic acid (A) and 100% acetonitrile (B). The column oven was operated at 35°C. About 1 µL of sample was injected onto a Thermo Scientific C18 column (Acclaim™ Polar Advantage II, 3 × 150 mm, 3 µm particle size) and subjected to the following gradient using an UltiMate 3000 UHPLC system (Dionex) HPLC system: Protocol 1 – 0.4 mL/min flow rate, 0-3 min – 5% B, 3-10 min – 80% B, 10-15 min – 80% B and 15-22 min – 5% B; Protocol 2 – 0.3 mL/min flow rate, 0 min – 2% B, 0-22 min – 28% B, 22-22.5 min – 40% B, 23-25 min – 95% B, and 25-30 min – 5% B; Protocol 3 – similar to Protocol 2, except X-bridge HILIC column (3.5 µm particle size, 150 × 4.6 mm) was used; and Protocol 4 – 0.3 mL/min flow rate, 0-1 min – 0% B, 1-5 min – 20% B, 5-20 min – 100% B, 20-25 min – 100% B, 25-26 min – 10% B, and 26-35 min – 0% B. The gradient parameter in Protocol 1 was based on Bruker's Gradient System Default method. The latter protocol was improvised from the default protocol. Sodium formate solution was injected as external calibration standard in the void volume of each chromatographic run.

High resolution mass spectrometry (MicroTOF QIII Bruker Daltonic, Germany) was carried out using ESI source in positive and negative ion modes, with the following settings: -capillary voltage: 4500 V; nebulizer pressure: 1.2 bar; drying gas: 8 L/min at 200°C. The mass range was at 50-1000 m/z. The MS data were processed through Data Analysis 4.1 software (Bruker Daltonics, Bremen, Germany). Relevant features were extracted using the Find Molecular Features algorithm and Dissect algorithm for deconvolution. The dissect algorithm for deconvolution combines all ions with a similar chromatogram profiles to one compound. Number of peaks and elution patterns were assessed from chromatograms generated. The proposed compounds were determined by online databases of ChEBI (Chemical Entities of Biological Interest), KEGG (Kyoto Encyclopedia of Genes and Genomes) and METLIN using the CompoundCrawler function in DataAnalysis. Metfrag would be used if no match was found from databases above.

RESULTS AND DISCUSSION

OPTIMIZATION OF LC-TOF-MS PROTOCOLS - FLOW RATES, GRADIENTS, OVEN TEMPERATURES, AND COLUMNS

Four separate protocols for LC-ToF-MS were assessed and optimized using metabolites extracted from maize inoculated with and without *S. parasiticum* through solid- and liquid-state cultivation. However, only results on the number of peaks based on fungal metabolites extracted using liquid-state cultivation were summarized in Table 1. The metabolites were extracted using 60% cold methanol supplemented with 1% formic acid. Based on the fungal metabolites extracted from liquid-state cultivation, protocol 2 gave the highest number of peaks at 24, followed by protocol 4 with 22 peaks, and lastly protocols 1 and 3 (less than 15) (Table 1). Protocol 2 produced a better chromatogram and more distinct basement compared to protocol 4 (Data not shown). The gradient steps in protocol 2 were designed to be much shallower with a longer gradient time and a lower flow rate to maximize the chromatographic peak capacity compared to protocol 1 and protocol 4. For protocol 3, the used of more polar column, X-bridge HILIC column (3.5 µm particle size, 150 × 4.6 mm) was employed to retain more polar compounds since many studies report the success of HILIC in improving the peak shape and enhanced sensitivity with ESI-MS detection (Gray et al. 2013). However, protocol 2 still produced better results based on the number of peaks detected compared to protocol 3. The number of peaks detected were much lower in HILIC column suggesting the extracts contains less polar compounds. Therefore, protocol 2 was selected for further optimization. Among the three solvents tested, namely acetonitrile, water and cold methanol amended with formic acid, the latter solvent yielded the highest number of peaks (Figure 1).

TABLE 1. Four different protocols of LC-MS studied and number of peaks. *Protocol 1 to 4 is referring to optimization of the LC-MS protocols, namely flow rates, gradients, oven temperatures, and columns

Protocols*	Number of peaks
Protocol 1	14
Protocol 2	24
Protocol 3	11
Protocol 4	22

**Protocol 1* – 0.4 mL/min flow rate, 0-3 min – 5% B, 3-10 min – 80% B, 10-15 min – 80% B, and 15-22 min – 5% B; *Protocol 2* – 0.3 mL/min flow rate, 0 min – 2% B, 0-22 min – 28% B, 22-22.5 min – 40% B, 23-25 min – 95% B, and 25-30 min – 5% B; *Protocol 3* – similar to Protocol 2, except X-bridge HILIC column (3.5 µm particle size, 150 × 4.6 mm) was used; and *Protocol 4* – 0.3 mL/min flow rate, 0-1 min – 0% B, 1-5 min – 20% B, 5-20 min – 100% B, 20-25 min – 100% B, 25-26 min – 10% B, and 26-35 min – 0% B

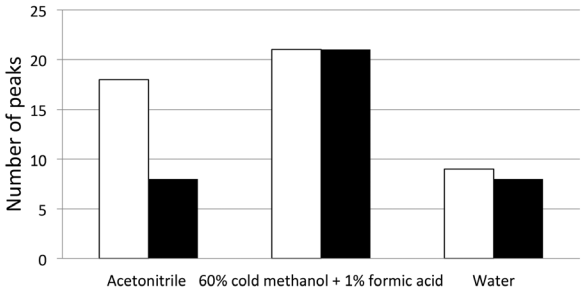

FIGURE 1. Number of peaks retrieved from liquid chromatography mass spectroscopy (LC-ToF-MS) for the samples with three different solvents. White and solid black bars refer to fungal metabolites extracted through liquid- and solid-state cultivation, respectively

OPTIMIZATION OF LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROSCOPY (LC-TOF-MS) - POSITIVE AND NEGATIVE POLAR

The putative metabolites and their respective retention times and M/Z values obtained through LC-ToF-MS with positive and negative ionizations were summarized in Table 2. These metabolites were sugars, alkaloids, amino acids, sugar alcohols and flavonoids. Negative polar ionization was observed to yield three more compounds (Table 2). The use of both positive and negative ionizations covered more wide-range of metabolites than the use of single polarity. Several metabolites were observed only at positive mode and some were detected in negative mode (Lei et al. 2011). Terpenoid and flavonoids founds in this study showed higher sensitivity in the positive ionization as compared to negative mode. These compounds such as arjunone, ligustroside, quercetin 5,7,3',4'-tetramethyl ether 3-rutinoside, benzal chloride and 1-Isothiocyanato-7-(methylsulfinyl)heptane were reported to have antioxidant, antifungal, anti-inflammatory and antimicrobial properties (Ferreira et al. 2010; Lim et al. 2017; Madla et al. 2012; Srivastava et al. 2001; Stupar et al. 2014).

OPTIMIZATION OF THE SOLVENTS FOR METABOLITES EXTRACTION

The effect of extraction solvents on the number of metabolites extracted from *S. parasiticum* was assessed. Fungal metabolites were initially extracted with absolute methanol, but the methanol extracts yielded fewer than 10 peaks (Data not shown). In a previous preliminary study, *S. parasiticum*-inoculate maize methanol extracts had greater growth inhibition in *G. boninense* compared to ethyl acetate extracts (Unpublished data). Therefore, methanol was selected as one of the extraction solvents. The extraction efficiency of a given solvent is evaluated by the number of peaks observed in the chromatogram. Among the three solvents tested, 60% methanol with 1% formic acid was the best in extracting metabolites from solid and liquid cultures of *S. parasiticum* compared to acetonitrile and water (Figures 1 & 2).

Similarly, Madla et al. (2012) showed that methanol was the suitable solvent for metabolite extraction of white-rot Basidiomycete, *Phanerochaete chrysosporium*, for many advantages such as good extraction efficiency and good recovery of metabolites. Recent study showed that the methanol extract of *Toona sinensis* leaves exhibited the highest inhibitory activity against *G. boninense*. Methanol is capable of extracting antifungal compounds due to the presence of hydroxyl and methyl group in methanol that could bind to polar, semi-polar and non-polar metabolites (Elfirta et al. 2018). In future, fractionation of methanol extract could be performed to identify bioactive compounds of *S. parasiticum*. Based on the comparative study on different solvents, 60% methanol amended with 1% formic acid was adopted for all other optimizations. There was no difference in term of number of peaks detected for both liquid- and solid-state cultivations (Figure 3).

TABLE 2. Putative compounds with their respective M/Z (H+) and retention time (min) at positive and negative ionizations for LC-MS

Positive ionization			Group of compounds	Negative ionization			Group of compounds
M/Z (H+)	Retention time (min)	Putative compound		M/Z (H-)	Retention time (min)	Putative compound	
236.079	1.9	N-Glycalyl-d-mannosaminolactone	Sugar	158.9795	1.7	Benzal Chloride	
147.0745	2.3	D-glutamine	Amino acid	145.0613	2.3	D-glutamine	Amino acid
130.0491	2.3	4-oxoproline	Amino acid	146.0465	2.4	Isoglutamate	Amino acid
204.1205	2.4	acetylcarnitine		181.072	2.5	D-manitol	
118.086	2.4	valine	Amino acid	570.1766	2.5	unknown	
183.0854	2.5	D-mannitol	Sugar alcohol	388.1199	2.6	6-acetonyldihydrosaguinamine	
345.1332	2.5	arjunone	flavonoids	387.1156	2.6	Fructoselynine 6 phosphate	
525.1976	2.5	Ligustroside	Terpenoid	549.1709	2.7	Asn-Trp-Cys-Glu	
685.2322	2.6	Citbismine c	Alkaloid	711.2242	2.8	Glucoliquinitin aproside	
325.1108	2.6	unknown		665.2161	2.8	Bifurcose	Sugar
847.2855	2.6	2-o-b-D-xylopyranosyl-L-arabinose		873.2772	2.8	unknown	
873.2772	2.8	Quercetin 5,7,3',4'-tetramethyl ether 3-rutinoside	Flavonoids	218.0685	3.6	1-Isothiocyanato-7-(methylsulfinyl) heptane	
				191.01968	4.9	Oxalosuccinic acid	Organic acid
				191.0196	4.9	Citric acid	
				606.0815	10.8	UDP-N-acetyl-D-mannosamine	Sugar

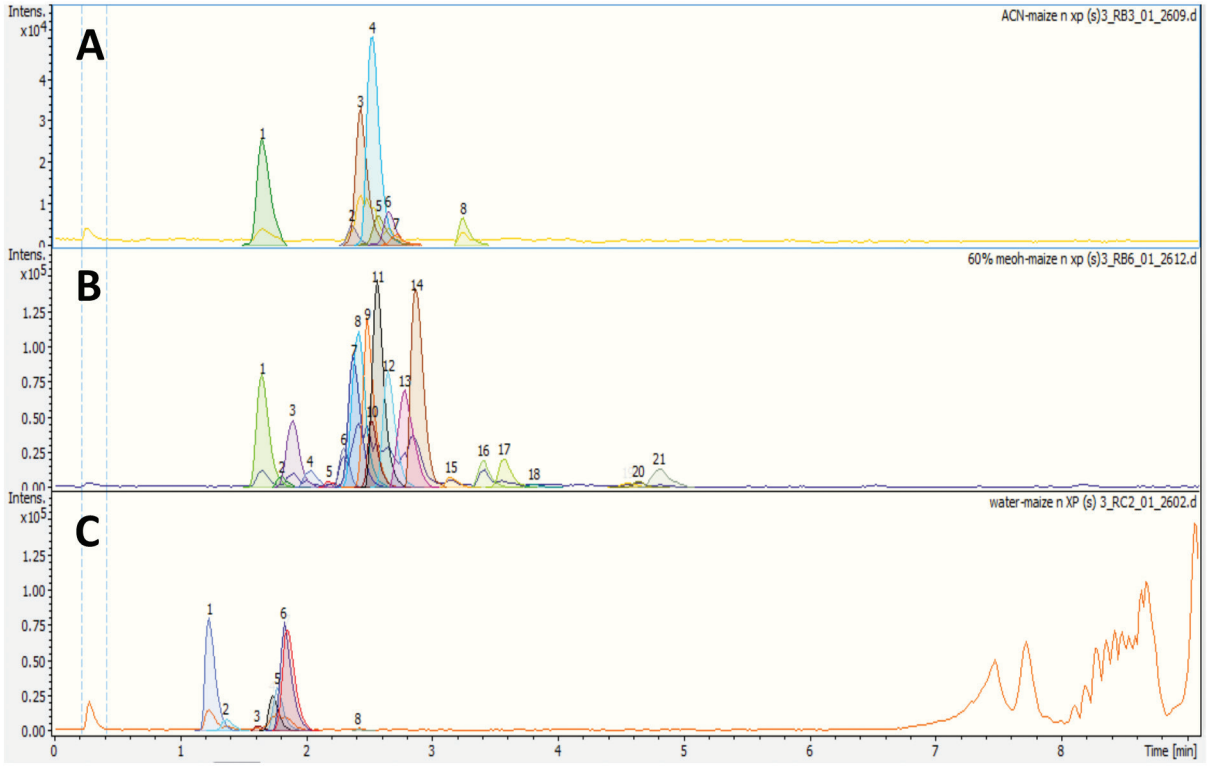

FIGURE 2. Chromatograms generated from metabolite extracts yielded from *Scytalidium parasiticum*-inoculated maize (solid-state cultivation) with three different solvents – acetonitrile (A), 60% methanol + 1% formic acid (B), and water (C)

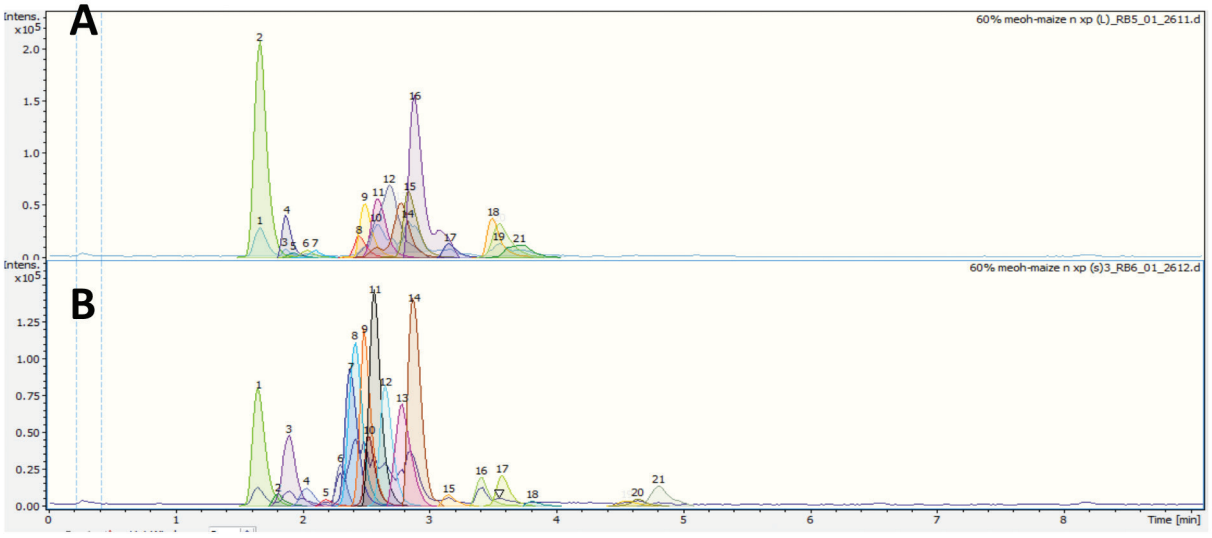

FIGURE 3. Chromatograms for metabolites extracted using 60% methanol with 1% formic acid from *Scytalidium parasiticum*-inoculated maize through liquid- (A) and solid- (B) state cultivations

OPTIMIZATION OF SAMPLES PREPARATION AND PROCESSING - WITH AND WITHOUT GRINDING AND SONICATION

Grounded samples, namely *S. parasiticum*-inoculated and non-inoculated maize yielded the highest numbers of peaks compared to non-grinding samples (Table 3). Grinding tissues in a liquid N_2-cooled mortar and pestle is a common method in breaking up tissues and cells, thus improving extraction efficacy. However, this method is laborious. In the future, homogenisation by an electric tissue homogeniser can be used considering speed and ease of extraction that this method can offer (Lin et al. 2007). Similar observations were recorded in sonicated samples (*S. parasiticum*-inoculated maize) with higher number of peaks compared to non-sonicated samples (Table 3). Ultrasonic-assisted extraction is a more useful approach

TABLE 3. Sample preparations and processing methods, and number of peaks

Sample preparation and processing	Samples	Number of peaks
Grounded *	Maize only[ǂ]	33
	Maize with Sp.[#]	27
Non-grounded	Maize only	17
	Maize with Sp.	18
Freeze-thawed**	Maize with Sp.	21
Non-freeze-thawed	Maize with Sp.	21
Sonicated	Maize with Sp.	30
Non-sonicated	Maize with Sp.	26

* Grinding refers to samples were grounded in Waring® blender prior to solvent extraction
** Freeze-thawing practice refers to samples in solvent were subjected to frozen with liquid nitrogen and thawing on ice
[ǂ] Maize only refers to non-inoculated maize substrates
[#] Maize with Sp. refers to *Scytalidium parasiticum*-inoculated maize substrates

than conventional solvent extraction method. It had been reported to improve recovery of metabolites with lower solvent consumption and had been used for the extraction of sugars, oil and polysaccharides (Gil-Chávez et al. 2013).

SAMPLES STORAGE AND DEGRADATION

Extracted metabolites samples were stored in -20°C for 2 weeks prior to metabolomics analysis with LC-ToF-MS. Degradation of the existing compounds or metabolites were observed. Once the extracts were stored in -20°C for 2 weeks, there was very low number of peaks and intensity of peak recorded (Data not shown). Therefore, the extracted samples were either stored in -80°C or analyzed within the first few days after metabolites extraction. It is crucial to store samples extracted in dark and at a very low temperature to avoid degradation. However, it is possible that some metabolites tend to degrade at low temperature due to oxidation (Pinu & Villas-Boas 2017).

CONCLUSION

We found that *S. parasiticum* in both liquid- and solid- state cultivation gave higher number of peaks in chromatograms when extracted with 60% methanol with 1% formic acid in combination with homogenisation methods such as ultrasonication and grinding. Further, some putative metabolites produced by *S. parasiticum* may have antifungal and antioxidant properties that merit future investigation. Current findings will be useful for future metabolomics studies on bitrophic *Scytalidium-Ganoderma* or *Scytalidium*-plant and tritrophic *Scytalidium-Ganoderma*-plant interactions, as well as screening for potential antifungal compounds to manage *G. boninense*.

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