

PRINCIPAL COMPONENT ANALYSIS (PCA) EVALUATION OF LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) DATASETS OF *Ganoderma boninense* INTRACELLULAR METABOLITES

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ABSTRACT

Liquid chromatography-mass spectrometry (LC-MS) has become a powerful analytical technique for studying broad coverage of chemical datasets describing complex biological systems and events. In order to interpret the underlying information in such datasets, multivariate analysis method such as principal component analysis (PCA) is crucial for multiple sample comparisons and multivariate data reduction. PCA has been used for evaluation of large-scale datasets derived from LC-MS analysis of fungal metabolites for many applications. Therefore, in this study, we describe on PCA as a descriptive tool to cope with large LC-MS datasets of intracellular metabolites of oil palm basal stem rot (BSR) fungal pathogen, *Ganoderma boninense* from in vitro liquid culture system. The results revealed a classification and grouping of *G. boninense* intracellular metabolites according to time trend, where the primary metabolites, i.e. glucose, gluconic acid, mannitol and malic acid were found differentially expressed in *G. boninense*. The presented findings suggest that the PCA model provides a general approach for handling, analysis and interpretation of large LC-MS datasets to reveal time-dependent changes of intracellular metabolites that may indicate *G. boninense* developmental process in vitro.

Keywords: *Ganoderma*, liquid chromatography-mass spectrometry, multivariate analysis, PCA.

Received: 2 June 2020; **Accepted:** 14 September 2020; **Published online:** 20 November 2020.

INTRODUCTION

A range of technical approaches have been used for the analysis of metabolites in biological samples including liquid chromatography-mass spectrometry (LC-MS). The technological advancement of LC-MS has been extensively reviewed elsewhere (Hayden *et al.*, 2019; Yuan *et al.*, 2019; Robison *et al.*, 2018) and increasingly become leading-edge instrumental developments due to its high sensitivity for the

detection of metabolic signatures in the analysis of pathogen (Chen *et al.*, 2019; Tugizimana *et al.*, 2019). LC-MS analysis generates information-rich multivariate datasets comprising of samples and variables that provide insight into the relationship of metabolites and changes in the status of complex biological systems and events. To interpret the underlying information within the complex LC-MS datasets towards reliable conclusions, it is of great importance that a practical and organised statistical modelling tool is applied.

The multivariate statistical analysis and data mining approaches for pattern discovery have facilitated information retrieval from the vast amount of biological data (Ivosev *et al.*, 2008; Jonsson *et al.*, 2005; Holmes and Antti, 2002). Principal component

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analysis (PCA) is a popular dimension reduction method to reduce high-dimensional data into a low-dimensional subspace component (DesRochers *et al.*, 2020; Gotthardt *et al.*, 2019; de Falco *et al.*, 2019; Diana and Tommasi, 2002). The information needs to be interrogated in a way that enables scientists to make a prediction and increases the interpretability of the large datasets, at the same time, minimising the loss of important information. Furthermore, PCA provides knowledge on the nature of the variables and assigns a large number of variables to a smaller number of groups that can be more readily visualised and understood (Ivosev *et al.*, 2008).

Ganoderma boninense is a well-recognised basidiomycete fungal pathogen associated with oil palm basal stem rot disease (Chong *et al.*, 2017) that causes significant economic losses by reducing the yield of fresh fruit bunches. The ability to generate and mine large-scale datasets of biological molecules, *i.e.* proteins, metabolites combined with recent advances in high-throughput instrumentation analysis and data mining tools may have a profound effect on the systems-level understanding of the pathogenicity of *G. boninense* (Othman *et al.*, 2019). Multivariate statistical analysis and data mining such as PCA has been used to evaluate large-scale datasets derived from LC-MS analysis of fungal metabolites for many applications, *i.e.* differentiation of virulent and avirulent species, assessment of metabolic behaviour in different culture environments and evaluation of metabolic difference in spores and mycelia (DesRochers *et al.*, 2020; Siless *et al.*, 2018; Son *et al.*, 2018; Liu *et al.*, 2015). Harnessing the complexity of the metabolomics data derived from the various analytical platforms remains as major challenges in metabolomics. To the best of our knowledge, up to now, there are no studies reported on the statistical evaluation and data mining of large-scale datasets from the *G. boninense* pathogen.

In that perspective, our study aimed to focus on multivariate statistical evaluation using PCA to cope with the large-scale LC-MS datasets from the *G. boninense* intracellular metabolites. This approach is important for a robust and reliable comparison of *G. boninense* intracellular metabolites measured over time using statistical strategy applied to the complex LC-MS datasets.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile, methanol and acetic acid of high performance liquid chromatography (HPLC) grade were purchased from Merck, Germany. Difco™ potato dextrose agar (PDA) and Difco™

malt extract broth (MEB) were purchased from Becton, Dickinson and Company (BD), USA. Ammonium phosphate monobasic [(NH₄)H₂PO₄], ammonium phosphate dibasic [(NH₄)₂HPO₄], potassium phosphate monobasic (KH₂PO₄), magnesium sulphate heptahydrate MgSO₄·(7H₂O), iron (III) sulphate heptahydrate (FeSO₄·7H₂O), zinc sulphate heptahydrate (ZnSO₄·7H₂O), sodium molybdate dihydrate (NaMoO₄·2H₂O), copper (II) sulphate pentahydrate (CuSO₄·5H₂O), manganese (II) chloride tetrahydrate (MnCl₂·4H₂O), 2-N-morpholinoethanesulphonic acid (MES) and glucose (≥99.5%) were purchased from Sigma-Aldrich, USA. Water was purified by a Milli-Q system (Millipore, USA).

Fungal Material Preparation and Liquid Culture Condition

Ganoderma boninense isolate PER71 was obtained from the Plant Pathology and Biosecurity Unit, Malaysian Palm Oil Board, Bangi, Selangor, Malaysia. *G. boninense* liquid culture protocol was previously described by Rees (2006). *G. boninense* was grown on Difco™ PDA (Becton, Dickinson and Company, USA) plate and incubated at 27 ± 1°C for eight days. A plug of 9.5 mm of *G. boninense* was harvested from eight days old culture on PDA plate. Biological replicates for each *G. boninense* were grown by inoculating one plug from each six individual PDA plates into 50 ml of MEB (Becton, Dickinson and Company, USA) in 175 cm³ Nunclon™ cell culture flask (ThermoFisher Scientific, USA). A total of six mycelial plugs were grown in the cell culture flask at 27 ± 1°C for six days. After six days, the *G. boninense* mycelial plugs were then collected on sterile filter paper, washed three times with sterile distilled water and inoculated into the 50 ml of growth medium supplemented with carbon and nitrogen sources in 175 cm³ Nunclon™ cell culture flask (ThermoFisher Scientific, USA). The growth medium consisted of: 0.9 g (NH₄)H₂PO₄; 2.0 g (NH₄)₂HPO₄; 1.0 g KH₂PO₄; 0.5 g MgSO₄·(7H₂O); 1 ml of 100x trace elements (1.11 g FeSO₄·7H₂O, 5.75 g ZnSO₄·7H₂O, 0.09 g NaMoO₄·2H₂O, 0.1 g CuSO₄·5H₂O and 0.21 g MnCl₂·4H₂O in 200 ml distilled water); 3.9 g 2-N-morpholinoethanesulphonic acid (MES) pH 5.5; 5.0 g glucose in 1 litre distilled water. The culture was grown at 27 ± 1°C. The *G. boninense* intracellular metabolites were sampled over time, on Days 2, 4, 6 and 8 for analysis.

Metabolite Extraction

The *G. boninense* mycelia containing intracellular metabolites were freeze-dried for two days using a FreeZone® Freeze Drier System (Labconco, USA). About 0.1 g of the powdered

mycelia was dissolved in 5 ml of methanol (Merck, Germany), vortexed for 1 min and sonicated for 15 min. The mixture was then centrifuged at $89 \times g$ for 15 min at 25°C. The supernatant was collected and the pellet was re-extracted with 5 ml of methanol, vortexed for 1 min, sonicated for 15 min and then centrifuged at $89 \times g$ for 15 min at 25°C. The supernatant was collected and pooled with the previous supernatant and dried under a nitrogen stream. The extract was dissolved in 3 ml of methanol (Merck, Germany) and filtered through a 0.2 μm cellulose acetate Minisart syringe filter (Merck, Germany). The filtered extracts were stored at -20°C before analysis. An aliquot of 10 μl was subjected to LC-MS analysis.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Ganoderma boninense intracellular metabolites were separated using the Ultimate 3000 HPLC (Thermo Scientific, USA), where the separation was achieved on a Reversed-Phase Acclaim™ Polar Advantage II (C18 4.6 \times 250 mm length, 5 μm particle size) (Thermo Scientific, USA). With a gradient elution program at a flow rate of 1.0 ml min⁻¹ and an injection volume of 5 μl , the chromatographic separation was performed at 37°C. The mobile phase A was an aqueous solution containing 0.1% (v/v) acetic acid (Merck, Germany) and mobile phase B was acetonitrile (Merck, Germany) containing 0.1% (v/v) acetic acid (Merck, Germany). The gradient program was started at 5% of B and increased linearly to 60% of B for 22 min. To minimise the carry-over between injections, the column was washed for 5 min and equilibrated for 2 min. Blank water injection was applied in-between the sample injection. All samples and replicates were injected simultaneously as one batch in random order to distinguish between technical and biological variations.

For the MS analysis, the HPLC system was coupled to a MicrOTOF-Q™ quadrupole-time-of-flight (QTOF) mass spectrometer (Bruker Daltonik, Germany) with an electrospray ionisation (ESI) interface operating in negative ion mode and controlled by the HyStar Application version 3.2 software (Bruker Daltonik, Germany). The column effluent was set at 1.0 ml min⁻¹. A split ratio of 1:4 was used, so the flow arriving at the detector was 250 μl min⁻¹. Nitrogen was used as nebulising gas at 4.1 bar and 9.0 litre min⁻¹ flow rate. The temperature and voltage of the capillary were set at 200°C and + 3.5 kV, respectively. The full MS scan covered the mass range of 50-1000 m/z . Tandem mass spectrometry (MS/MS) parameters were set to improve mass fragmentation, with collision energy varying from 15-35 eV.

LC-MS Data Evaluation and Principal Component Analysis (PCA)

LC-MS data evaluation was performed with DataAnalysis version 4.2 for chromatogram analysis and ProfileAnalysis version 2.1 (Bruker Daltonik, Germany) for PCA. Compounds in the raw LC-MS data were extracted using MS algorithm version 3.0, sensitivity 99%, absolute intensity of 1000 and minimum peak valley of 10% under Find Compounds – Chromatogram module in DataAnalysis version 4.2, for comprehensive detection of all compounds in LC-MS run. PCA was performed based on bucket tables of the MS data. The data was integrated from 2.0-22 min and 50.5-999.5 m/z in time- and m/z -buckets using time alignment parameters and spectral background subtraction in the advanced bucketing approach. The multivariate statistics, PCA, was performed where data were Pareto scaled. The LC-MS data were then normalised to the sum of bucket values for an analysis, which considered the total intensity in analysis and suitable for unknown samples. A full cross and test set validations were applied to validate the PCA model. In ProfileAnalysis version 2.1 (Bruker Daltonik, Germany), the SmartFormula module was used to process the accurate mass data of molecular ions. The list of possible elemental formulas that use CHNO algorithm such as minimum/maximum elemental range, electron configuration, ring-plus double bond equivalents and comparison of the theoretical with measured isotope patterns was provided in the module. Compounds identified were categorised based on MSI (Metabolomics Standard Initiative) (Sumner *et al.*, 2007).

RESULTS AND DISCUSSION

One of the challenges in LC-MS metabolomics studies is data analysis and metabolite annotation, *i.e.* identifying biological molecules from mass spectral data. The highly sensitive LC-MS platform allows the simultaneous detection of multiple analytes combined with data processing workflow using ProfileAnalysis analysis including peak picking, filtering, normalising and statistic analysing (Mamat *et al.*, 2018; Veeramohan *et al.*, 2018; Mazlan *et al.*, 2018; Tahir *et al.*, 2016). In this study, the number of peaks detected from *G. boninense* intracellular metabolites were increased over time, until Day 6 and decreased at Day 8 (Figure 1). The metabolites measured over time were further analysed using PCA.

Validation of *Ganoderma boninense* Intracellular Metabolite PCA Model

PCA was used as a processing tool to enable robust interpretation of large-scale data from the

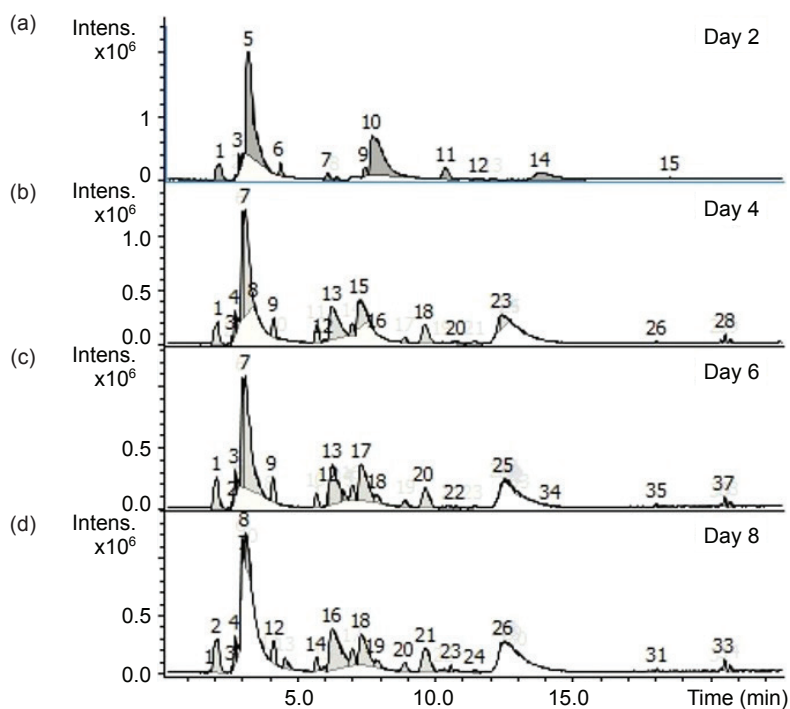


Figure 1. The liquid chromatography-mass spectrometry (LC-MS) base peak chromatograms (BPC) of *Ganoderma boninense* extracellular metabolites at each time point of Day 2 (a), 4 (b), 6 (c) and 8 (d). The highest number of 37 peaks were detected at Day 6, followed by Day 8 with 33 peaks, Day 4 with 28 peaks and Day 2 with 15 peaks.

G. boninense intracellular metabolites. Moreover, validation methods are crucial for the evaluation of model performance and prediction ability (Diana and Tommasi, 2002). The rule of cross validation (CV) technique is to test the significance and to correct the confidence limits and was applied to the PCA model for the *G. boninense* intracellular metabolites. As shown in Figure 2, full CV was applied to compare the predicted values with the actual ones. The influence plot of a PCA model for the *G. boninense* intracellular metabolites showed similar results before (a) and after (b) validation, where, no outlying behaviour outside the quadrant. This indicated the significance of the model at 95% confidence interval.

Besides, a test set validation of a PCA model was also applied to check the stability of the current model. Test set validation is suitable for large datasets (>50) (Kos *et al.*, 2003). Figure 3 shows the influence plot of the original *G. boninense* intracellular metabolites PCA model (Figure 3a) and the new model after validation (Figure 3b). The new model described the test set analyses well, where the basic appearance of the scores plot did not change. This indicated the initial set of analyses all together resulted in a good and valid model.

***Ganoderma boninense* Intracellular Metabolite PCA Model**

Large multivariate datasets generated from the LC-MS analysis of *G. boninense* intracellular

metabolites require pattern recognition techniques that filter the important information from different time points and detect compounds that contribute to the groupings according to time trend. In this study, therefore, we employed an unsupervised method such as PCA to summarise the multidimensional data as well as to capture most of the variance in the datasets. PCA is a mathematical projection technique designed to extract many variables, displays and ranks the variance in a reduced data matrix (Jolliffe and Cadima, 2016). Furthermore, PCA extracts relevant statistics on the distribution of samples and group characteristics (Lazar *et al.*, 2015).

PCA is one of the most important and powerful methods in chemometrics (Bro and Smilde, 2014). Besides, it can also identify the effects of technical variation in the analysis of metabolic profiles, which is crucial for data quality evaluation in metabolomics studies (Gika *et al.*, 2014; Yin *et al.*, 2013). Following the pre-processing LC-MS data of the *G. boninense* intracellular metabolites, PCA allowed a descriptive evaluation of the samples distribution in order to assess the data quality and to identify natural groupings, patterns and outliers as explained and described in other reports (Tugizimana *et al.*, 2019). The samples presented in the PCA scores plot in Figure 4a showed close clustering between data points for each time interval. This indicated a good quality, stability, reliability and reproducibility of the analyses. The scores plot displays the relationship between

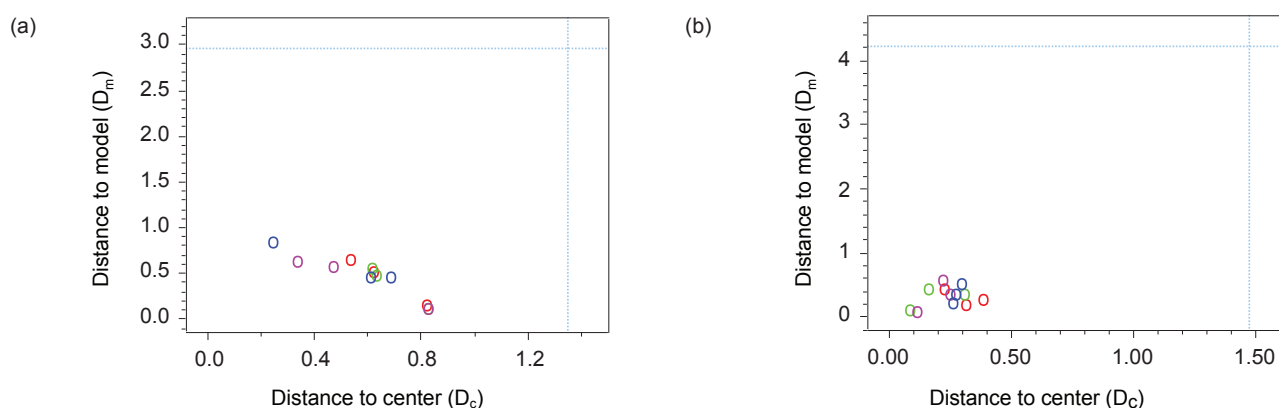


Figure 2. Full cross validation of *Ganoderma boninense* intracellular metabolite principal component analysis (PCA) model. Influence plots (a) before and (b) after validation show no outlying behaviour outside the quadrant and indicated the significance of the model at 95% confidence interval.

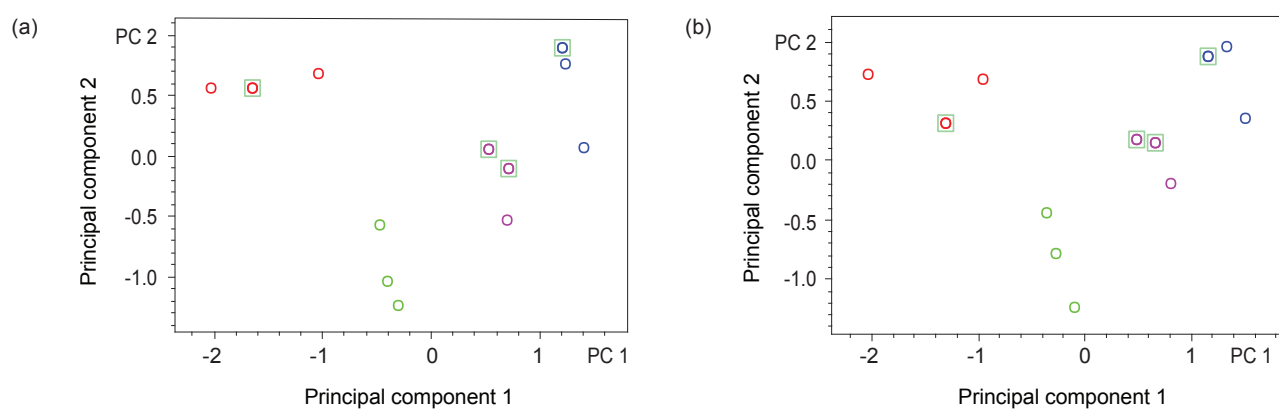


Figure 3. Test validation of *Ganoderma boninense* intracellular metabolite principal component analysis (PCA) model. The scores plot shows (a) the selection of four test set analyses before (b) and after test set validation. The new PCA model (b) described the test set analyses well, where the basic appearance of the scores plot did not change and resulted in a good and valid model.

G. boninense intracellular metabolites harvested at the different time intervals of Days 2, 4, 6 and 8.

Furthermore, in PCA, a linear transformation of the metabolic features in specific directions is called principal components, where they are taken seriously in explaining the most variation of the first component, followed by the second component, *etc.* (Jolliffe and Cadima, 2016; Alonso *et al.*, 2015). In this study, as shown in the scores plot, PC1 and PC2 explained 87.6% of the total variance present in the dataset. The first principal component summarises the global dataset since it captures most of the dataset variability in feature/metabolite patterns based on their similarity (Alonso *et al.*, 2015). In our results, PCA models revealed distinct time trend groupings in the *G. boninense* intracellular metabolites. Here, clear sample groupings indicate dynamic changes occurring in *G. boninense* developmental process *in vitro*, and observed as time-dependent stages, related by the time trend which was revealed by PC analyses (Figure 4b). PCA, as a standard model to deal with high-dimensional and complex metabolomics data, proved to uncover time-trends

in a dataset (Tugizimana *et al.*, 2019). The PCA loadings plot (Figure 4b) represents 375 buckets (time and *m/z*) included in the PCA model. In this model, the four buckets that are far away from the central cloud are responsible for the variance within the dataset; (1) rT 3.6 min, *m/z* 179.0560, (2) rT 4.5 min, *m/z* 195.0514, (3) rT 2.9 min, *m/z* 181.0719 and (4) rT 6.6 min, *m/z* 133.0144.

Ganoderma boninense Intracellular Metabolite Signatures

From the PCA loadings plot analysis, four metabolites that were located far away from the central cloud, which were responsible for the variance within the dataset were selected and identified as differentially expressed metabolites. Metabolites were assigned as (1) glucose (*m/z* 179.0560), (2) gluconic acid (*m/z* 195.0514), (3) mannitol (*m/z* 181.0719) and (4) malic acid (*m/z* 133.0144) based on SmartFormula (Bruker Daltonik, Germany), comparison against KEGG database and tandem MS (MS/MS) (Table 1). MS/MS spectra and

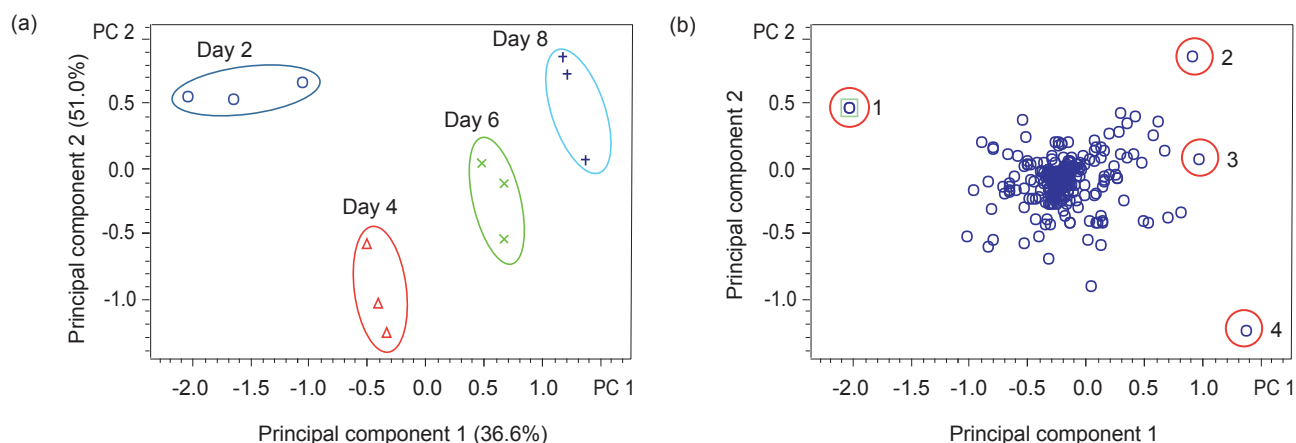


Figure 4. Principal component analysis (PCA) of the *Ganoderma boninense* intracellular metabolite LC-MS data. Scores plot (a) and loadings plot (b) of negative mode data (Pareto-scaled) from intracellular samples. Each data point in the PCA scores plot represents an analysis included in the PCA model. A 2-component model, explaining 87.6% of the total variation in the Pareto-scaled data at 95% confidence level, from each time point at Days 2, 4, 6 and 8. A clear time-related trend is observed.

chemical structures of all identified metabolites were presented on Figures 5-8. According to the PCA loadings plot, glucose showed relatively high abundance at Day 2 while gluconic acid, mannitol and malic acid showed relatively high abundance at Days 4, 6 and 8, respectively. Bucket statistics plot in the ProfileAnalysis enables a quick visual comparison of the differentially expressed metabolites according to time trend (Figure 9).

The differentially expressed intracellular metabolites in *G. boninense*, which were classified as primary metabolites, *i.e.* sugar and organic acid, play significant and essential roles in the growth, reproduction and development of the fungus. These metabolites are associated with the rapid initial growth phase and show maximum

production at the end of the phase (Andersen, 2014; Wisecaver *et al.*, 2014; Sanchez and Demain, 2008). Mannitol, for example, is the most abundant soluble carbohydrates and plays a key role as carbon storage/ carbohydrate reserve within the mycelium of fungi (Patel and Williamson, 2016; Wiemken, 2007; Lewis and Smith, 1967). However, mannitol has recently been hypothesised to have a major role in fungal pathogenicity (Patel and Williamson, 2016).

CONCLUSION

Ganoderma boninense is an economically important pathogen in oil palm where a holistic and detailed understanding of the pathogen biology may

TABLE 1. PROPOSED DIFFERENTIALLY EXPRESSED INTRACELLULAR METABOLITES OF *Ganoderma boninense* AT DAYS 2, 4, 6 AND 8

No.	rT (min)	Metabolite	KEGG ID	MSI	Molecular formula	Monoisotopic mass (g mol ⁻¹)	<i>m/z</i> measured	<i>m/z</i> theoretical	Error (ppm)	MS ² fragments
1.	3.6	Glucose	C00031	1	C ₆ H ₁₂ O ₆	180.06	179.0560	179.0561	0.7	161.0433, 131.0372, 119.0374, 101.0252
2.	4.5	Gluconic acid	C00257	2	C ₆ H ₁₂ O ₇	196.06	195.0514	195.0510	-2.0	177.0398, 159.0282, 129.0180
3.	2.9	Mannitol	C00392	2	C ₆ H ₁₄ O ₆	182.08	181.0719	181.0718	-0.6	161.0465, 119.0334, 113.0217, 101.0222
4.	6.6	Malic acid	C00711	2	C ₄ H ₆ O ₅	134.02	133.0144	133.0142	-1.4	115.0017, 89.0189

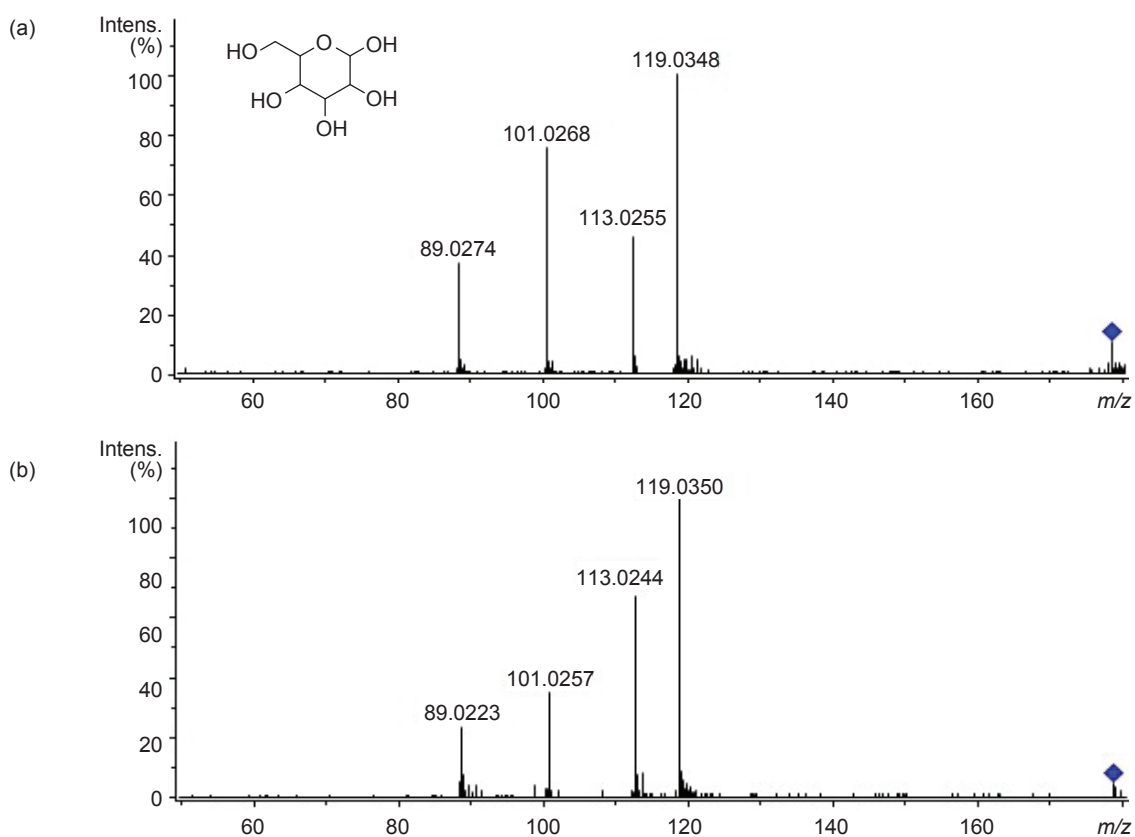


Figure 5. Negative ion mode mass spectrometry/mass spectrometry (MS/MS) spectra of glucose (m/z 179.0560, $C_6H_{12}O_6$) at a collision energy of 20 eV. Similar MS/MS spectra of (a) glucose in *Ganoderma boninense* mycelia sample and (b) glucose reference standard.

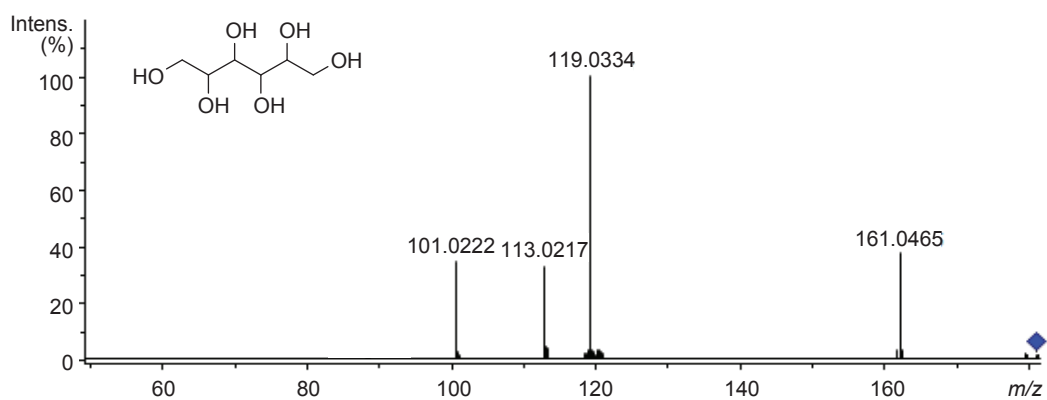


Figure 6. Negative ion mode mass spectrometry/mass spectrometry (MS/MS) spectrum of mannitol (m/z 181.0719, $C_6H_{14}O_6$) at a collision energy of 35 eV.

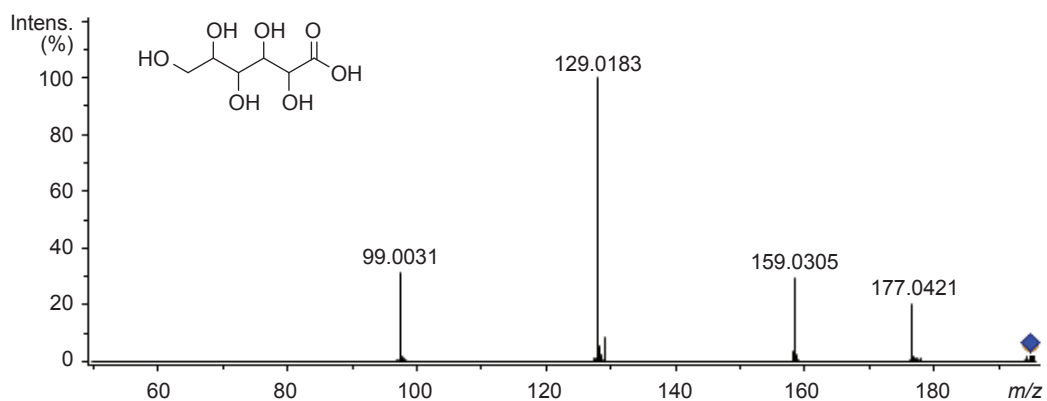


Figure 7. Negative ion mode mass spectrometry/mass spectrometry (MS/MS) spectra of gluconic acid (m/z 195.0514, $C_6H_{12}O_7$) at a collision energy of 15 eV.

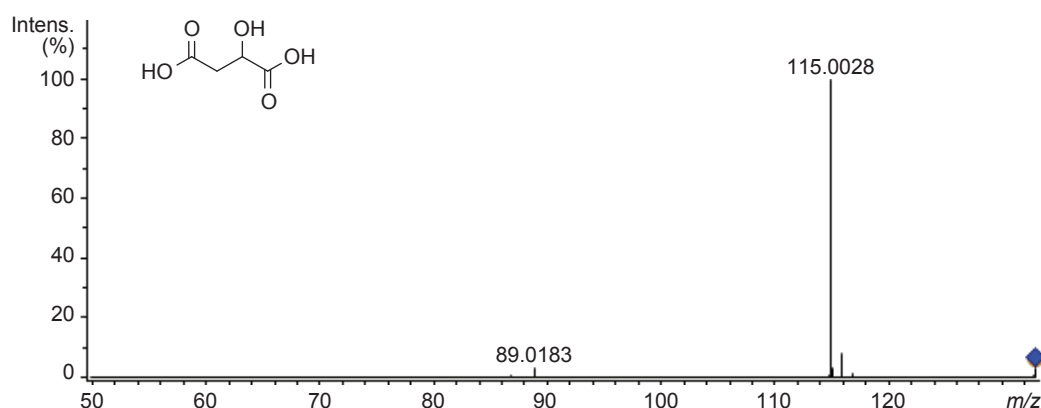


Figure 8. Negative ion mode mass spectrometry/mass spectrometry (MS/MS) spectra of malic acid (m/z 133.0144, $C_4H_6O_5$) at a collision energy of 15 eV.

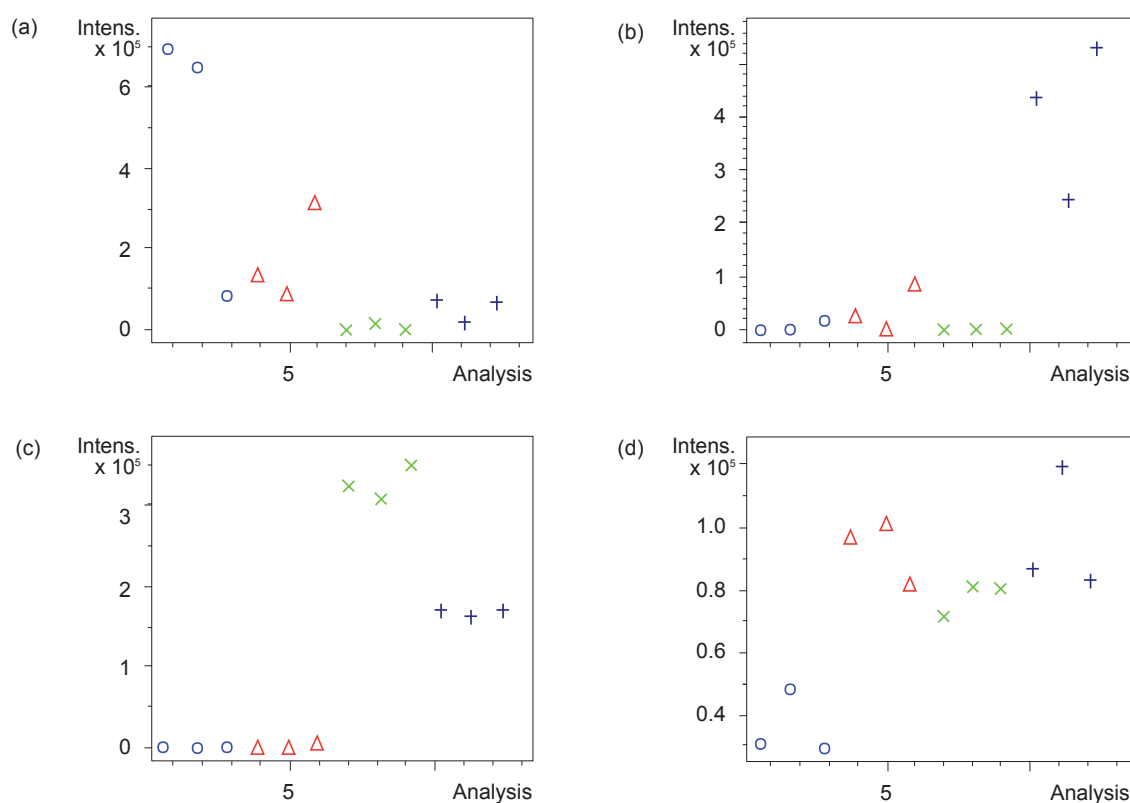


Figure 9. Bucket statistic plot of four differential metabolites observed in the loadings plot of principal component analysis (PCA) (● for Day 2, Δ for Day 4, × for Day 6 and + for Day 8). (a) Glucose ($C_6H_{12}O_6$, m/z 179.0560), (b) gluconic acid ($C_6H_{12}O_7$, m/z 195.0514), (c) mannitol ($C_6H_{14}O_6$, m/z 181.0719) and (d) malic acid ($C_4H_6O_5$, m/z 133.0144).

significantly improve targeted disease control and management strategies. The present study provides a necessary step for uncovering molecular events during the developmental process of *G. boninense* using LC-MS and multivariate statistical data analysis, particularly PCA. Albeit the interpretation of the large and complex LC-MS datasets of fungal metabolites is frequently challenging, our studies have demonstrated that PCA provides a robust and reliable comparison of temporal intracellular metabolite changes of *G. boninense*. This may indicate the dynamic changes

occurring in *G. boninense* developmental process, *in vitro*. Furthermore, this information is of key importance that can complement the available transcriptomics and proteomics data of *G. boninense*, towards further understanding the *G. boninense* pathogenicity.

ACKNOWLEDGEMENT

The authors would like to thank the Director-General of MPOB for permission to publish this

article. We would also like to thank the members of the Proteomics and Metabolomics Unit, Advanced Biotechnology and Breeding Centre (ABBC), MPOB for their technical support and invaluable contribution. We acknowledge the support and assistance of Jayanthi Nagappan, Bioinformatics Unit, ABBC.

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