Investigation of metabolites produced by *Magnaporthe oryzae* during appressorium development using ^1H NMR metabolomics approach

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Abstract. This study was aimed to determine metabolites produced by *Magnaporthe oryzae* and identify metabolic changes during appressorium development. Appressorium development were induced *in vitro* and subjected to ^1H NMR spectroscopy for metabolites production and multivariate data analysis. PCA, PLS-DA and OPLS-DA were used to profile metabolite production throughout appressorium development. There were 43 metabolites identified putatively and PCA showed differences of metabolites production between mycelium and appressorium development. Metabolites that were significantly produced ($p < 0.05$) during appressorium development including isocitrate, isobutyrate, lysine, glutamate, succinate, tyrosine, choline, glycerol, xylose, mannose, sucrose, tryptophan, butyrate, leucine, isoleucine, valine, ethanol, methylmalonate, threonine, lactate, alanine, arginine, 4-aminobutyrate, homoserine, glucose, mannitol and glucitol. Glycerolipid, carbohydrates and amino acids metabolisms showed to be highly involved during appressoria development. This study revealed metabolites produced by *M. oryzae* during appressoria development *in vitro* as first metabolomics data using ^1H NMR approach.

Keywords: appressorium, *Magnaporthe oryzae*, metabolomics, Proton Nuclear Magnetic Resonance (^1H NMR)

INTRODUCTION

Rice is important food source to mankind. More than 3 billion people worldwide rely on rice as their daily diet resulting in increase of global food demand from the expanding world population (Skamnioti & Gurr, 2009). Despite of its high demand, rice is often affected by various biotic and abiotic stresses including insect pests, diseases, salinity, iron toxicity and drought (Zhang & Xie, 2014). One in particular, the rice blast disease has seriously destroyed rice plants which caused by recalcitrant fungal pathogen, *Magnaporthe oryzae*. The effect of this disease on

rice production is severe enough to be considered as threat to world's rice production with estimation of total loss up to 10-30% each year (Talbot, 2003). *Magnaporthe oryzae* is a hemibiotrophic fungus which initially lives on rice plants symptomless (biotrophic) and begin to associate with host by destroying plant cells over time (necrotrophic) (Fernandez & Orth, 2018). *Magnaporthe oryzae* undergoes complex developmental stages including development of appressorium. Appressorium is a unicellular, dome-shaped structure adhered on leaf surface

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and progressively creates turgor pressure by accumulation of solutes as a mechanical force whereby presses on leaf cuticle (Fernandez & Orth, 2018). Once the appressorium ruptures the leaf cuticle, penetration peg beneath the appressorium emerges right through a small pore to form a primary hyphae and develops into invasive hyphae within the rice tissue (Ebbole, 2007). Integrated control measures have been implemented to decrease the severity of rice blast but complete eradication is still unachievable. Methods including screening and developing resistant cultivar (Azizi *et al.*, 2015), chemical control, biological control, nutritional management have been introduced to manage the disease (Miah *et al.*, 2017) but comprehensive study is also needed to understand *M. oryzae* physiological processes. In doing so, this study focus on metabolites produced by *M. oryzae* to provide information on key metabolites responsible during appressoria development hence revealing underlying biochemical mechanisms of *M. oryzae*. This also provide opportunities to develop effective control measures. Previous study on *M. oryzae* metabolites obtained from infected leaf unveiled several metabolites such as alanine, malate, glutamine and cinnamate exhibited largest changes after 24 h post inoculation (Jones *et al.*, 2011). Several genetic manipulation studies in *M. oryzae* indicated arginine and lysine were essential for appressoria formation and pathogenicity (Chen *et al.*, 2014; Zhang *et al.*, 2015). Despite these findings, there is no clear metabolic profile involving appressoria development of *M. oryzae in vitro* has been reported using metabolomics approach considering appressoria is important infection structure for plant infection. As metabolomics has emerged as powerful tools for analysis of metabolites from living samples (Clarke & Haselden, 2008; Kim *et al.*, 2011), we consider ¹H NMR as reliable approach to determine metabolites profile from *M. oryzae* during appressorium development. ¹H NMR able to provide robust data analysis with simple sample preparation techniques and producible chemometrics approach. Therefore, the objectives of this study were to determine metabolite produced by *M. oryzae* appressorium development and examine metabolites associated with changes between 0 h, 8 h and 24 h. Our study provided new insight of metabolites produced by

M. oryzae and metabolic pathways involved during appressorium development were discussed.

MATERIALS AND METHODS

Maintenance of fungal strain and growth condition

Fungus *Magnaporthe oryzae* strain Guy11 was used in this study. Fungus was routinely maintained on complete media (CM) agar plate. CM per litre consists of: 50 mL of 20X nitrate salts, 1 mL of 1000X trace elements, 10 g of glucose, 2 g of peptone, 1 g of yeast extract, 1 g of casamino acids and 1 mL of vitamin solutions as described by Talbot *et al.* (1993). In addition, 100 µg/mL antibiotic solution of streptomycin and penicillin was added to prevent bacterial growth. Fungus was incubated at 26°C until fully grown.

Preparation of fungal biomass

Conidia of *M. oryzae* were harvested by scraping the surface of fungus using glass spreader in sterile distilled water. The conidia were filtered through layers of miracloth (Merck, USA) and centrifuged at 10 000 rpm for 8 min. The conidia were snap frozen in liquid nitrogen and freeze dried. For induction of appressoria, conidia were grown on plastic coverslips (Cole-Parmer, USA) and appressoria formation was observed (Soanes *et al.*, 2012). Conidia were harvested after 8 h and 24 h inoculation, frozen in liquid nitrogen and lyophilized. Samples were stored at -80°C until further analysis. For mycelial sample preparation, *M. oryzae* from agar plate were transferred to CM broth, homogenized and incubated for 36 h at 200 rpm, 26°C. Subsequently, mycelia in CM broth were filtered by miracloth, rinsed with sterile distilled water and immediately frozen in liquid nitrogen and lyophilized (Soanes *et al.*, 2012).

Metabolite extraction

Six replicates of 0 h, 8 h, 24 h and mycelium samples were weighed up to 30 mg (dry weight) in 2 mL microcentrifuge tubes prior to metabolite extraction. Metabolites were extracted based on Kim *et al.* (2010) procedure. Lyophilized fungus was dissolved in 1:1 ratio of solvent which were 0.75 mL methanol-d₄ (CD₃OD) and 0.75 mL of KH₂PO₄ buffer (1.232 g of KH₂PO₄ mixed with

100 mL of D_2O and 10 mg TSP and pH adjustment to 6.0 using 1.0 M NaOD). Fungal extracts were vortexed for 1 min at 26°C and disintegration of sample were carried out using ultrasonic bath for 20 min. The extracts were then centrifuged at 26°C for 10 min, 13 000 rpm to obtain clear supernatant. Lastly, 600 μL of supernatant were transferred to 5 mm-size NMR tube (Norell, Sigma- Aldrich, Canada) for NMR analysis.

Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were acquired by NMR spectrometer of Varian NMR System 500 MHz equipped with OneNMR probe (Varian, CA, USA). Sample was loaded into NMR spectrometer and solvent deuterium oxide (D_2O) was set to lock the spectrometer frequency. NMR measurement was conducted at 25°C with automated shimming along with parameter of spectral width 8012.8 Hz, relaxation delay (RD) 2.0 s, 64 number of scans and pulse width (PW) 8.60 μs (90°). TSP was used as internal reference to calibrate chemical shift peak at 0.0 ppm. Presaturation was carried out to remove mountain-like water peak appeared on the spectra by weak radiofrequency irradiation.

Spectral processing and statistical analysis

Spectra obtained from ^1H NMR were manually pre-processed before subjected to binning and multivariate data analysis. Spectra collected in “.fid” file were exported to “.cnx” file so that phase and baseline correction, pH calibration and chemical shape indicator (CSI) adjustment could be performed using Chenomx Processor NMR software (Chenomx NMR Suite 5.1 Professional, Canada). Spectral binning was generated using Chenomx Profiler software. NMR data matrices contained 250 variables (columns) and 24 observations (rows). Bin spectrum line were normalized to total intensity of spectra (Wiklund, 2008). Data was imported to SIMCA-P 13.0 (Umetrics, Umeå, Sweden) for data mining of multivariate statistical analyses. The dataset was mean-centred and Pareto scaled before undergo Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) analyses. Then, data was further analyzed using Orthogonal Partial Least Squares-Discriminant

Analysis (OPLS-DA). Model validation for OPLS-DA was performed by permutations given value of R^2Y and Q^2Y (Eriksson *et al.*, 2006).

Metabolites identification and pathways analysis

Metabolites were identified from web databases- Human Metabolome Data Base (HMDB) <http://www.hmdb.ca>, Biological Magnetic Resonance Data Bank (BMRDB) <http://www.bmrdb.wisc.edu>, Spectral Database for Organic Compounds (SDBS) https://sdb.sdb.aist.go.jp/sdb/sdb/cgi-bin/direct_frame_top.cgi and Chenomx Profiler which using peak fitting and similarity matching from identified compound database. Metabolites identification was also verified using reported literature and articles. Heat map, hierarchical clustering and pathways analysis were generated using a free access online software Metaboanalyst 4.0 (<https://www.metaboanalyst.ca>) (Chong *et al.*, 2019). Pathways analysis were constructed based on pathway database from KEGG (Kyoto Encyclopedia of Genes and Genomes) (<https://genome.jp/kegg/>) and literature search to illustrate the proposed metabolic pathway involved during appressoria development.

RESULTS AND DISCUSSION

^1H NMR spectral analysis

Determination of metabolites produced by *Magnaporthe oryzae* during appressorium development was assessed at 3 consecutive time-points including 0 h, 8 h, 24 h as well as from mycelium. ^1H NMR spectra representative of each group with labelled metabolites was shown in Figure 1. The 43 metabolites were identified putatively from various source of metabolomics web databases, peak fitting software (Chenomx Profiler) and previous reports. From the spectra, abundant primary metabolite signals could be observed from δ 0-5.5 ppm whereas weak signals appeared at aromatic region, δ 5.6- 8.5 ppm. There were several differences of peak intensities exhibited by each of *M. oryzae* development stage from 0 h, 8 h, 24 h and mycelium (m).

Figure 1. ¹H NMR spectra of 500 MHz spectrometer representative from each group of m: mycelia, 0 h: 0 hour, 8 h: 8 hours and 24 h: 24 hours. TSP (3-Trimethylsilylpropionic acid) used as internal reference at 0.00 ppm. Metabolite assignments were according to chemical shift multiplicities: 1 leucine; 2 valine; 3 isoleucine; 4 butyrate; 5 ethanol; 6 lactate; 7 alanine; 8 arginine; 9 lysine; 10 4-aminobutyrate; 11 methylmalonate; 12 threonine; 13 homoserine; 14 glutamate; 15 succinate; 16 glutamine; 17 isocitrate; 18 methionine; 19 citrate; 20 2-oxobutyrate; 21 aspartate; 22 asparagine; 23 cysteine; 24 choline; 25 glucose; 26 xylose; 27 mannose; 28 glycerol; 29 mannitol; 30 glucitol; 31 tyrosine; 32 2-hydroxyvalerate; 33 malate; 34 uridine diphosphate (UDP) glucose; 35 2-phosphoglycerate; 36 sucrose; 37 cytosine; 38 fumarate; 39 3-hydroxyphenylacetate; 40 tryptophan; 41 xanthosine; 42 xanthine; 43 adenine.

Multivariate data analysis

1. PCA and PLS-DA analysis

Multivariate analysis able to monitor metabolite changes in *M. oryzae* during appressoria development. Principal component analysis (PCA) was performed as primary evaluation of model to identify similarity pattern of spectra from metabolic profile, cluster and outliers. Similar metabolites profiles will group close together while different profiles are further apart (Aliferis & Jabaji, 2010). In Figure 2(a), first component, PC1 described maximum variation of data, 59% followed by PC2 which is 25%. In account to PC1, mycelia located at positive quadrant meanwhile 0 h, 8 h and 24 h groups belong to negative quadrant. Similarity was shown

in 0 h, 8 h and 24 h appressoria group as it situated near to each other but differ compared to mycelia. This showed that similar metabolites profile between 0 h, 8 h and 24 h but distinct metabolite profile when compared to mycelia. The R^2 and Q^2 are cross validation values calculated by SIMCA software for estimation the goodness of the model fit the data and goodness of predictive (Jones *et al.*, 2011). R^2 value must higher than Q^2 and $Q^2 > 0.5$. In this PCA model, R^2 is 0.886, Q^2 is 0.814 which indicated reliable model. Figure 2(b) represents metabolites distribution from PCA component 1 and 2. Glucitol, mannitol, methylmalonate, choline, lactate and threonine contributed the most to influence group clustering of developmental stages between mycelium and appressoria (0 h, 8 h and 24 h).

Figure 2. PCA score plot of ^1H NMR (a) loading scatter plot (b). Score plot shows data matrices of appressoria development stage at 0 h, 8 h, 24 h and m: mycelium from crude *Magnaporthe oryzae* extract.

Data was further analyzed using PLS-DA to reveal hidden variables that mainly focus on class discrimination (Eriksson *et al.*, 2006). PLS-DA is supervised method where class of data was set as known membership to detect the most influential metabolites responsible for changes between group of appressoria development stage (Aliferis & Jabaji, 2010). Figure 3(a) showed score plot of

PLS-DA model indicated Q^2 value of 0.733 with $R^2X = 0.898$ and $R^2Y = 0.902$ (4 PLS components). PLS-DA revealed same pattern as PCA and was validated using permutations with R^2 and Q^2 values of 0.261 and -0.442 respectively which confirmed its validity. When vegetative mycelia showed distinct metabolites profiles compared to appressoria development (0 h, 8 h and 24 h), this

indicate that *M. oryzae* might activated certain process or metabolism during appressoria formation that is not required during mycelia non-infection stage. This result is consistent with findings from Soanes *et al.* (2012) that demonstrated gene expression of mycelium is highly distinct from appressorium development on artificial surface. Study by Jones *et al.* (2011) demonstrated metabolic profile of *M. oryzae* from infected rice leaves which resulted in wide range of metabolites however it may also have been contributed by the host as response to counteract fungal pathogen. Hence, particular activation of genetic regulation and metabolism might occur during infection process in *M. oryzae* appressoria development *in vitro* which do not occur in mycelium resulted in difference of metabolites produced. In terms of morphology, mycelia are network of hyphae that do not acquire infective structure like appressoria, which critically important to infect plants. Mycelia also have been cultivated in rich media in contrast to nutrition limitation condition in appressoria. On the other hand, appressoria formation and development is regulated by complex genetic control for example like glycerol turgor generation (Thines *et al.*, 2000), cell wall biogenesis and melanin deposition in the appressorium (Tucker *et al.*, 2004), autophagy of conidia after 8-16 h (Veneault-Fourrey *et al.*, 2006), thus suggesting that throughout the processes, appressoria must undergo metabolic pathways involved in fungal infection distinct from mycelium. Furthermore, cell wall in appressorium is more specialized cell wall in contrast to vegetative mycelia (Tucker *et al.*, 2004).

Metabolites that influence group discrimination among appressoria development and mycelium could be evaluated by referring to VIP [Figure 3(b) and Table 1]. VIP is variable of important projection depicts most important variable sufficient for class discrimination and VIP value more than 1 indicates significant variable that responsible for clustering of 0 h, 8 h, 24 h and mycelia (Wang *et al.*, 2015). From 43 identified compound, 30 metabolites were significantly influenced separation of group based on PLS-DA model (Table 1). Significant high levels of sugars namely glucitol, mannitol, mannose, glucose were showed in loading column component 1 negative w^*c [1] representing mycelia group Figure 3(c). Positive w^*c [1]

revealed metabolites belong to appressoria group (0 h, 8 h and 24 h). Metabolites that were significantly influenced appressoria group discrimination were caprate, butyrate, isobutyrate, isocitrate, glutamate, ethanol, isoleucine, methylmalonate, homoserine, sucrose, 3-hydroxyphenylacetate and tryptophan when compared to mycelia group.

2. OPLS-DA analysis of metabolites during appressoria development

To evaluate possible key metabolites that produced by *M. oryzae* during appressoria development, we analyzed OPLS-DA model after PLS-DA model to determine discriminants in each distinct time point of 0 h, 8 h and 24 h. External validation using permutations was performed based on PLS-DA model (3 components) with given values of $R^2 = 0.419$ and $Q^2 = -0.252$ confirmed its validity [Figure 4(b)]. OPLS-DA model resulted $R^2X = 0.913$, $R^2Y = 0.98$ and $Q^2 = 0.861$ with percentage of variation $PC1 = 32\%$ and $PC2 = 7.09\%$. Clear discrimination was obtained from OPLS-DA as shown in Figure 4(a) and metabolites discriminant for each group was given in Table 2 corresponded to PC1 and PC2. Appressoria is formed after 4-8 h indicated caprate, lysine, glutamate, succinate, tyrosine, choline, glycerol, mannose, sucrose, 3-hydroxyphenylacetate and tryptophan as possible key metabolites produced during appressorium formation. During 24 h, metabolites produced were amino acids such as leucine, alanine, isoleucine and tyrosine suggesting amino acid metabolism is required during appressorium maturation.

High number of amino acids production were observed during appressorium development. The relationship of amino acids biosynthesis with fungal infection have been demonstrated by genetic studies and considered to be essentially important for growth and pathogenicity involving *M. oryzae*. Biosynthesis of isoleucine involved threonine deaminase, *MoIh1* as catalyst to convert threonine into NH_2 and ketobutyrate acid and mutants with disrupted *MoIh1* exhibit abnormality in conidia, low conidia production and retarded growth of invasive hyphae (Du *et al.*, 2014). Lysine also involved in infection process of *M. oryzae* and we speculate that lysine production was higher during 24 h appressoria maturation

because lysine might be used for invasion penetration process involving invasive hyphae. This is because mutants lack of lysine biosynthesis showed restricted growth of invasive hyphae that unable to penetrate and therefore failure of plant infection (Chen *et al.*, 2014). These findings indicated that amino acids are essential for pathogenicity in *M. oryzae*.

Glycerol was mainly produced at 8 h and 24 h and it is one of the compound necessary for turgor generation. This result highlighted the importance of glycerol as solutes and it is potentially synthesized by glycogen and lipid degradation from conidia reserves (Thines *et al.*,

2000). Glycerol is being retained in the appressorium with the help of melanin deposition that prevent glycerol from moving out during turgor generation (de Jong *et al.*, 1997). The accumulation of glycerol caused enormous pressure forced by the appressorium to rupture host cuticle (Ebbole, 2007). Triacylglycerol also involved in direct glycerol generation by the aid of enzyme triacylglycerol lipase (EC 3.1.1.3) and triacylglycerol degradation may produce fatty acids and then undergo β -oxidation to produce acetyl-CoA for glyoxylate cycle and gluconeogenesis (Thines *et al.*, 2000).

Figure 3. Metabolites that influenced group clustering between 0 h, 8 h, 24 h and mycelium were analyzed using PLS-DA model. (a): Score plot of PLS-DA model (b): VIP plot (c): Loading column plot of PLS-DA component 1.

Table 1. List of metabolites that had VIP value > 1. VIP (variable influence on projection) described most important variables contributed to group separation from the spectra.

Metabolites	Chemical shift, ppm (experimental)*	Chemical shift, ppm (reference)	VIP value
caprate	0.82, t	0.85, t	1.35215
butyrate	0.86-0.89, t	0.88, t	1.38303
leucine	0.94-0.982, m	0.948, t, J=5.897	2.3579, 1.4277
	1.65-1.78, m	1.70, m	
	3.70-3.75	3.722, m	
isoleucine	1.01-1.03, d	0.997, d, J=7.001	1.55759
	0.90-0.945, t	0.926, t, J=7.414	1.17659
	1.20-1.24, m	1.248	1.94767
	1.94-2.00, m	1.968	
	3.65-3.66, d	3.969, d, J=3.661	
valine	0.98-1.00, d	0.976, d, J=7.01	
	1.03-1.06, d	1.029, d, J=7.05	1.50668
	2.22-2.27, m	2.261, m	
isobutyrate	1.13, d	1.21, d, J=7.02	1.31314
ethanol	1.16-1.20, t	1.17, t	2.02955
	3.64-3.66, m	3.65, q	
methylmalonate	1.25-1.30, d	1.23, d, J=7.35	2.73365
threonine	1.30-1.325, d	1.316, d, J=6.585	2.28464
	4.23-4.28, m	4.244, m	
	3.578-3.592, d	3.575, d, J=4.867	1.79672
lactate	1.32-1.34, d	1.32, d, J=6.96	2.04243
	4.07-4.12, q	4.10, q, J=6.93, 6.93	1.66841
alanine	1.46-1.49, d	1.46, d, J=7.14	2.41359
lysine	1.48-1.58, m	1.46, m	1.64682, 1.45725
	1.68-1.78, m	1.71, m	
arginine	1.60-1.80, m	1.68, m	1.53455
4-aminobutyrate	1.85-1.92, m	1.90, m	1.08554, 2.20667
	2.26- 2.31, t	2.30, t	1.49633
	2.97-3.02, t	3.01, t	1.75358, 1.51406
glutamate	1.98-2.09, m	2.07, m	1.03904
	2.10-2.18, m	2.10, m	1.14777, 1.20167
	2.34-2.40, m	2.35, m	1.25701, 1.77525
homoserine	2.00-2.06, m	2.01, m, J=7.53	1.7537, 1.43104
glutamine	2.43-2.47, m	2.47, m	1.08422
isocitrate	2.50-2.60, m	2.46, m	1.18207
choline	3.20-3.21, s	3.189, s	2.8776
	3.49-3.51, m	3.507, dd, J=5.816, 4.16	
	4.02-4.06, m	4.056, m	
glucose	3.22-3.26, m	3.23, dd	1.54946
	3.36-3.40, m	3.39, m	2.17096
	3.40- 3.43, m	3.458, m	1.5578
	3.51-3.55, m	3.524, dd	
	5.23-5.25, d	5.223, d, J=3.677	
xylose	3.30, m	3.31, dd, J=10.41, 11.45	1.97973
	3.89-3.94, m	3.91, dd, J=11.54, 5.47	
	4.56-4.59, d	4.57, d, J=7.90	1.65946
	5.16-5.18, d	5.19, d, J=3.66	1.58364

mannose	3.57, t	3.584, t	
	4.85-5.05, d	5.127, d, $J=1.317$	2.87384, 1.95616
glycerol	3.50-3.56, m	3.525-3.577, m	1.73538, 1.84584
	3.62-3.66, m	3.618-3.670, m	2.62436
mannitol	3.70-3.74, m	3.729, m	2.63887, 2.136
	3.85-3.88, m	3.84, dd $J=11.87, 2.8$	2.16496
glucitol	3.78-3.82, m	3.764, m	2.40468
	3.80-3.85, m	3.838, m	3.22986
tyrosine	3.16-3.22, m	3.17, dd	1.76406
	3.05-3.09, m	3.024, dd	
	3.93-3.95, m	3.92, dd	
	6.82-6.86, d	6.87, m	
	7.15-7.19, d	7.17, m	
2-hydroxyvalerate	4.02-4.06, m		1.18991
	1.36-1.40, m		
sucrose	5.30-5.40, d	5.40, d, $J=3.89$	1.38538, 1.11956
3-hydroxyphenylacetate	6.76-6.80, m	6.79, m	
	7.24-7.28, m	7.26, t, $J=7.75$	1.29458
tryptophan	7.70-7.73, d	7.72, d	1.31902
	7.50-7.52, d	7.53, d	1.00097
	7.30-7.32, s	7.31, s	
	7.19-7.22, m	7.19, m	

*Experimental chemical shifts (ppm) were compared with reference chemical shifts from various published articles and online databases. Peak pattern indicated as: s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet and J represents coupling constant measured from J-resolved 2D NMR library. VIP values obtained from VIP plot generated from PLS-DA model.

Figure 4. Model of OPLS-DA revealed discriminant for each group of 0 h, 8 h and 24 h appressoria development. (a): score scatter plot of 0 h, 8 h and 24 h of appressoria development. (b): Model validation permutations plot.

Table 2. Discriminant of each group 0 h, 8 h and 24 h during appressoria development in *Magnaporthe oryzae* based from OPLS-DA loading column plot. Metabolites were classified based on metabolites cluster in component 1 and component 2.

0 h	8 h	24 h
isocitrate	caprate	butyrate
2-hydroxyvalerate	lysine	isoleucine
isobutyrate	glutamate	leucine
	succinate	valine
	tyrosine	ethanol
	choline	methylmalonate
	glycerol	threonine
	xylose	lactate
	mannose	alanine
	sucrose	lysine
	3-hydroxyphenylacetate	arginine
	tryptophan	4-aminobutyrate
		homoserine
		glutamate
		succinate
		tyrosine
		choline
		xylose
		mannose
		glucose
		glycerol
		mannitol
		glucitol
		sucrose
		3-hydroxyphenylacetate
		tryptophan

Concentration of metabolites were compared between mycelium, 0 h, 8 h and 24 h and visualized in heat map (Figure 5). Hierarchical cluster analysis (HCA) was calculated by average linkage clustering method and Euclidean distance measurement. Metabolites that highly produced in mycelium compared to appressoria group were sugars like mannitol, glucose, mannose, glucitol, 2-hydroxyvalerate (hydroxy fatty acids), and amino acids group, glutamine and 4-aminobutyrate. Glutamine level in appressoria development at 8 h and 24 h were observed low compared to mycelium. During 8 h to 24 h, glutamine levels supposedly to be in low concentration because it might involve in conserved Target of Rapamycin (TOR) signalling pathway that is negative-regulatory pathway (not

required pathway) during appressorium formation (Marroquin-Guzman & Wilson, 2015) in contrast with cAMP/PKA MAP kinase pathways that are positive-regulatory pathway (required pathway) for appressoria formation. Activation of TOR (inhibitor pathway) that coded by GATA transcription factor *ASD4* revealed mutants of deleted *ASD4* showed high expression of *GLN1* glutamine synthetase, thus producing high glutamine in the cells. High expression of *GLN1* caused high glutamine production that is unfavourable condition for appressoria therefore mutant of *ASD4* was unable to form appressoria *in vitro*. Thus, our result consistent with this finding on low glutamine production during appressoria development. However, the role of glutamine in *M. oryzae* is still unclear.

Figure 5. Heat map represents concentration of identified metabolites from binned ^1H NMR in account with VIP > 1 when comparing with group of mycelium, 0 h, 8 h and 24 h. Green colour indicates lowest level minimum -4 and red indicate highest level maximum 4.

3. Metabolic pathways involved during appressoria development

Metabolism occurred during appressoria development could be related to various metabolic pathways. ^1H NMR data of significant metabolites production were analyzed to suggest most significant pathways involved in *M. oryzae* appressoria development stage by using the metabolic pathway analysis, MetPa. MetPa has generated pathway impact factor which measures significance of metabolites in to be involved in most relevant pathways (Chong *et al.*, 2019). Figure 6 illustrates metabolisms that had impact factor greater than 0.1 which acceptable value (Azam *et al.*, 2017) and the highest impact factor observed was 0.45 indicated by amino acid

metabolism of alanine, aspartate and glutamate. The other metabolisms involved during appressoria development were starch and sucrose metabolism, butanoate metabolism, glycerolipid metabolism, arginine biosynthesis, glycine, serine and threonine metabolism, glyoxylate and dicarboxylate metabolism and arginine and proline metabolism. The relationship of metabolites produced by *Magnaporthe oryzae* during appressorium development were depicted in proposed metabolic pathways schematic diagram in Figure 7.

Glyoxylate cycle and tricarboxylic acid cycle (TCA) were crucial during appressoria development. The precursor of glyoxylate, isocitrate was required to produce malate,

converted into oxaloacetate and then acetyl-CoA (see Figure 7). Isocitrate production was increased at early stage of conidia (0 h) suggests it could be fed into TCA as well to produce succinate and oxalosuccinate consequently for production of glutamate, glutamine and lysine. Specific enzymes, isocitrate lyase (ICL) (Wang *et al.*, 2003) involved

in catalyzed isocitrate to produce glyoxylate and glyoxylate cycle was required for virulence in *M. oryzae* as shown by deletion of isocitrate lyase gene-encoding enzyme that had caused retardation of appressorium formation, conidia formation and cuticle penetration (Wang *et al.*, 2003).

Figure 6. Pathway analysis performed by the metabolic pathway analysis (MetPa) viewed all matched metabolisms according p value of pathway enrichment analysis and impact values of pathway topology analysis. It suggests most important metabolism during *M. oryzae* developmental stages based from identified metabolites of ¹H NMR data.

CONCLUSION

In this study ¹H NMR metabolomics was used to assess metabolite produced by *M. oryzae* during appressorial development. There were 43 metabolites identified from ¹H NMR spectra. Multivariate data analysis revealed metabolites pattern and metabolites that significantly changes ($p < 0.05$) during appressorial development including isocitrate, isobutyrate, butyrate, leucine, isoleucine, valine, ethanol, methylmalonate, threonine, lactate, alanine, arginine, 4-aminobutyrate, homoserine, mannitol, glucitol,

lysine, glutamate, succinate, tyrosine, choline, glycerol, xylose, mannose, glucose, sucrose and tryptophan. Metabolite production were evaluated using pathway analysis to predict underlying metabolism required for infection. Metabolic pathways that are highly involved during appressorial development of *M. oryzae* were alanine, aspartate and glutamate metabolism, sugar and sucrose metabolism, glyoxylate and dicarboxylate metabolism and glycerolipid metabolisms. Further investigation on these metabolisms will provide additional information on their roles in infection development and how it can be manipulated to aid disease management.

Figure 7. Schematic diagram of proposed metabolic pathways involved during appressoria development of *M. oryzae*. Metabolites differences produced by *M. oryzae* were highlighted in 3 colours; red- 0 h, green- 8 h and 24 and purple- 24 h. Multiple arrows in pathways represent series of reactions that were not shown. Metabolic pathways were analyzed using MetPa (Metaboanalyst.ca) and KEGG (Kyoto Encyclopedia of Genes and Genomes) database.

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CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

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