ASM Sc. J., 12, Special Issue 3, 2019 for ICST2018, 40-47

Differential Expression and Profile of Oil Palm Root-sterols Composition Related to *Ganoderma boninense* Infection

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Ganoderma boninense is a basidiomycetous fungus commonly associated with Basal Stem Rot (BSR) disease in oil palm (OP). The opportunistic pathogen colonizes OP through degradation of cell wall upon which the plant induces an array of metabolic changes for resistance or enhances susceptibility. Studying the cell wall-lipidomic profile could lead to the identification of important metabolites associated with *G. boninense* infection. Therefore, the aim of the study is to stimulate *G. boninense* infection at the primary site of infection, the roots cell wall, and to identify the sterol profile related with the infection at early and late stage. Oil palm seedlings were artificially infected (AI) with *G. boninense* and harvested after 3 and 6 months of incubation, regarded as first (T1) and second interval (T2) respectively. The OP cell wall-sterols were extracted and analysed using Gas Chromatography-Mass Spectrometry (GC-MS). The sterol profiles obtained were further analysed using Metaboanalyst 4.0. At interval stages, using a univariate analysis, 77 and 56 significant features were recognized between healthy and infected groups at T1 and T2 respectively. Based on the results form the multivariate analyses, ten metabolites, (Heptacosane, γ-sitosterol and β-sitosterol, stigmasterol, (24R)-ergost-4-en-3-one, octadecane, 1-iodo-, 5α-lanost-8-en-3β-ol, ergosterol, stigmastanol and campesterol) were selected as the discriminatory metabolites that were important in both intervals. These metabolites might be used as diagnostic biomarkers to identify infected palms at early stage.

Keywords: oil palm; cell wall; sterol; metabolite composition

I. INTRODUCTION

Advanced-stage of Basal Stem Rot (BSR) disease, caused by *Ganoderma* fungus is associated with high incidences of mortality and lower yielded palms. It has been reported that the economic loss caused by this pathogen is up to 500 million USD a year (Arif *et al.* 2011; Ommelna *et al.* 2012). While there no yet effective treatment measures for this disease, it is important that BSR disease is detected early for improved prevention, prognosis and for economical infection management. Recently, a number of novel techniques have been developed that aid with the early detection of *Ganoderma* (Ariffin and Idris, 1991; Lelong *et al.,* 2010; Abdullah *et al.,* 2011; Alexander *et al.,* 2014; Dayou *et al.,* 2014; Chong *et al.,* 2017). However, the

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cycle of BSR disease consists of a number of alternative and consecutive events thus making it difficult to be detected in early stages (Hushiarian *et al.,* 2013). During the pathogenesis, fungi posses an array of digestive enzyme as common features to pass the plant cell wall, an important physical barrier against pathogen. Subsequently host plant initiate defense barrier to prevent further colonization and infection of their host plants. There are multiple alterations in the wall-membrane lipid and lipid-derived molecules from the plant or the microbial organism involved during the plant-pathogen interaction (Siebers *et al.,* 2016). Lipids played a crucial role in various stages of the infection process, such as intracellular signaling and plant-host cross-talk. Lipid sterols have been shown to be involved in structural and signaling function, affecting the fate of fungi virulence against plants (Cassim *et al.,* 2018). A study by Rossad *et al.* (2010) reported that a fungal sterol ergosterol had shown to trigger plant innate immunity in sugar beet. In further study by Wang *et al* (2012) also confirmed the accumulation of plant sterol, stigmasterol in *Arabidopsis* leaves when challenged with *Pseudomonas syringae*. This suggests that sterols may also plausibly linked to metabolic pathways upon *G. boninense* attack await for discovery. The aim of the present study was to study the cell wall-sterol composition of oil palm roots during *G. boninense* infection and their change trends in the principal stages of *Ganoderma* infection in oil palm.

II. METHODOLOGY

A. Preparation of plant materials

Oil palm (OP) seedlings (eight-months old) were artificially inoculated with *G. boninense* following the method as described by Breton *et al.* (2006). The seedlings were first uprooted carefully and later kept seated on the *G. boninense*-inoculated RWB to ensure the roots were in direct contact with the inoculum. Twenty oil palm seedlings were subjected for *Ganoderma* inoculation, meanwhile another twenty oil palm seedlings without the inoculum were served as healthy controls. Extreme care was taken to minimize injury during inoculation and planting. All OP seedlings were placed and arranged in complete randomized blocks under netted plant house conditions with 70% of shade protection with regular watering. Destructive sampling of the seedlings were done at two intervals which first interval (3 months after AI) and second interval (6 months after AI). Ten inoculated and ten uninoculated (healthy) seedlings were assessed for each sampling time. Roots tissues from the seedlings were harvested and washed under tap water, soaked in 99% ethanol for a few seconds and air-dried. The success of *Ganoderma* infection was re-confirmed using *Ganoderma* Selective Media (GSM) (Ariffin and Idris, 1991) and undergone ergosterol analysis (Chong, 2012).

B. Extraction of roots cell wall-sterols

Oil palm root materials (100 g fresh weight) were lyophilized for 48 hours and homogenized using commercial blender until fine powder. Three g of roots samples were suspended in 50 mL acetate buffer (pH 4.6) and the cell walls were separated from soluble cystoplasmic fluid by centrifuging at 1,000 x g for 15 min. The pellet was washed extensively with 50 mL of acetate buffer, thrice and was lyophilized overnight. Thereafter, one g of cell wall materials were added to 50 mL centrifuge tubes along with 10 mL of solvent mixture (chloroform: methanol (2:1, v/v)). The tube was vortexed and agitated in temperature-controlled incubator shaker for 120 mins at 30˚C temperature. Then the tubes were centrifuged at 2000 rpm for 10 min to recover liquid phase. Solvent mixture were washed with 0.2 volume of miliQ® water and vortexed for a few seconds. The tubes were centrifuged again at 2000 rpm to separate into two phases. The aqueous upper phase was removed and the lower chloroform phase containing lipid was kept for further use. The extracts were later dried under a stream of nitrogen at room temperature.

C. Gas Chromatography-Mass Spectrometry analysis

The sterol was redissolved in dichloromethane and analyzed by gas chromatography-mas spectrometry (GC-MS, Agilent 5975C) using a J&W GC column (30 m x 250 μm×0.25 μm; Agilent). The injector temperature was maintained at 280˚C. The column temperature was

initially kept at 100˚C for 2 min and then increase in a rate of 10˚C/min up to 300˚C and held at this temperature for 25 min. Helium was used as a carrier gas at a flow rate of 0.9 mL/min. The mass spectrometer was operated in electron impact mode (70 eV). Data acquisition was performed in full scan mode from m/z 50 to 650 with a scan time of 0.5 sec.

D. Data processing and statistical analysis

GC-MS data file was converted into CDF format, and then directly processed using MZmine (version 2.2.3). The MZmine report table was exported into web based service for metabolomic data analysis: MetaboAnalyst 4.0 (http://www.metaboanalyst.ca.) for chemometric analysis. The data was pareto-scaled by mean centering and variance scaling to remove the offsets and adjust the importance of all metabolites to an equal level normalized the data. Pairwise comparison (univariate) within intervals was done separately to observe significant changes of sterol composition at specific stage of infection. In a second analysis (multivariate), groups (Healthy sample-interval 1 (H-T1), Infected sample-interval 1 (IN-T1), Healthy sample-interval 2 (H-T2) and Infected sample-interval 2 (IN-T2)) comparison was performed to discover potential biomarkers for infected tissue classification. Analysis of un-supervised and supervised such as principle component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed. Potential differential metabolites were selected according to the Variable Importance in the Projection (VIP) value and the metabolites with VIP > 1.2 and >1.5 from the first and second analysis respectively.

III. RESULTS AND DISCUSSION

The differential metabolites between different sample groups were first categorized according to the stage of infection. Univariate statistical analysis was performed on H-T1 and IN-T1 datasets (three replicates of each group) to determine affected sterol metabolites at first interval. Fold change (FC) at $\log_2 > 2$ analysis of the datasets identified 77 important features with p-value < 0.05. PCA analysis revealed that principal component 1 (PC1) showed maximum variation with 92.4% of total variance (Data not shown). In PLS-DA analysis, the components also showed the separation pattern similar to PCA analysis (Figure 1 (A)). PLS-DA VIP analysis identified five metabolites (VIP score > 1.2) significantly changed between IN-T1 and the H-T1 groups (Figure 1 (C)). In IN-T1 dataset, two metabolites (tetradecane and γ-sitosterol) and three metabolites (aminoacetone, ergosterol and stigmasta-5,7-dien-3β-ol) were down-regulated and up-regulated respectively in their levels. Metabolism of aminoacetone, a precursor of methylglyoxal may associated with the production of reactive oxygen species (ROS) (Kalapos, 2008) in response to *G. boninense* colonization, although there is little evidence that these routes occur in plants. In the plant cell, ROS can directly cause strengthening of host cell walls via lipids peroxidation and membrane damage (Lamb and Dixon, 1997; Montillet *et al.,* 2005). However, there is also evident that ROS are important signals in defense occur in conjunction with other plant signaling molecules (Levine *et al.,* 1994).

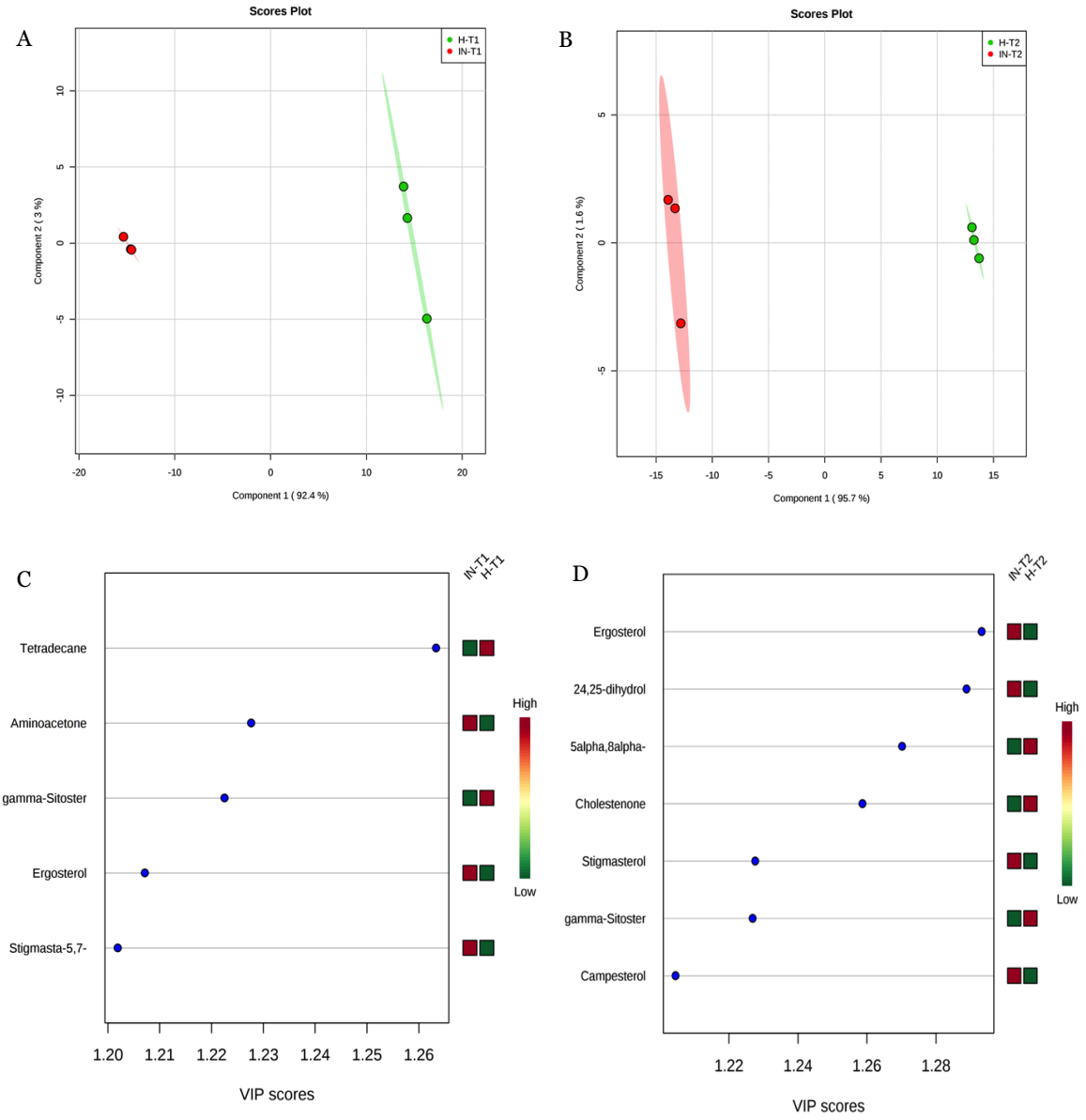

Figure 1. Partial least square-discriminant analysis (PLS-DA) of (A) IN-T1 vs. H-T1 and (B) IN-T2 vs. H-T2; Variable importance in projection (VIP) score plot selected from PLS-DA model with p > 0.05 and (VIP) scores > 1.2 of (C) IN-T1 vs. H-T1 and (D) I

Similarly, at second intervals, IN-T2 group was separated from H-T2 group. A total of 56 significant features were recognized (log_2 (FC) > 2 and *p*-value < 0.05). recognized (log_2 (FC) > 2 and *p*-value < 0.05). PCA (Data not shown) and PLS-DA (Figure 1 (B)) shows separation of the two samples along PC1 with 95.7% of total variance. According to the PLS-DA VIP-score plot, a total of seven variables (metabolites) with VIP scores > 1.2 were highly contributed to separation between groups (Figure 1 (D)). In IN-T2 datasets, four metabolites (Ergosterol, 24,25-dihydrolanosterol, stigmasterol and campesterol) were up-regulated and levels of 5α, 8α -epidioxy-24E-ethylidene-cholest-6-en-3β-ol,cholest-enone and γ-sitosterol were found to be substantially low in relative to H-T1. Using univariate analysis, IN-T1 *vs.* H-T1 and IN-T2 *vs.* H-T2 shared one metabolite, ergosterol that was up-regulated in infected samples at both intervals. The number of differential sterol metabolites in second interval decreased compare to first interval, suggesting higher amount of sterol in the first interval are associated with palm defense mechanisms. The changes

in sterol composition of roots CW due to *G. boninense*-infection may influence OP disease resistance and affect the outcome OP–*G. boninense* interactions.

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Multivariate statistical approach was carried out to identify if there were any common changes in sterol composition among infected (IN-T1 and IN-T2) and healthy (H-T1 and H-T2) groups at first and second intervals. One-way ANOVA was performed to recognize the significance of the sample cohorts analyzed, followed by post-hoc analyses using Fisher's LSD to identify the differences among the levels in the datasets. ANOVA analyses identified a total of 105 significant features with p-value < 0.05. PLS-DA and PCA analysis of the datasets were performed to study the variance in the data amongst the two components that were derived upon these analyses. PCA analysis reveals clear differences between infected and healthy palm at both first and second interval. The first two PC, PC1 and PC2 were responsible for major variation (70.7%) in this study groups (Figure 2 (A)). Metabolites contributing to this discrimination were identified using supervised PLS-DA ($Q^2 = 0.982$; $R^2 = 0.952$) (Figure 2 (B)). PLS-DA VIP score-plot identified 12 variables (VIP score > 1.5) with the highest contribution to this signature.

Further analysis using heatmap recognize a total of ten metabolites, three metabolites (Heptacosane, γ-sitosterol and β-sitosterol) significantly down-regulated while, seven metabolites (stigmasterol, (24R)-ergost-4-en-3-one, octadecane, 1-iodo-, 5α-lanost-8-en-3β-ol, ergosterol, stigmastanol and campesterol) were up-regulated in infected groups, indicating that the two groups could be separated based on these metabolites (Figure 2 (C)). Stigmasterol, stigmastanol, sitosterol, (24R)-ergost-4-en-3-one and campesterol are major phytosterols in plants and their abundances change drastically during abiotic and biotic stresses. Similar to this finding, Nusaibah *et al.* (2011) also found the abundance of these phytosterols *Ganoderma*-infected oil palm seedlings. Upon infection with pathogenic microbes, sitosterol converted into stigmasterol metabolite to induce resistance or enhance susceptibility. Accumulation of the phyrosterol stigmasterol during the *Arabidopsis thaliana-Pseudomonas syringae* interaction was also reported (Griebel and Zeier, 2010). Another study by Nusaibah *et al.* (2016) found decreased lipid level, β- and γ-sitosterol in susceptible oil palm progeny challenged with *G. boninense*, supporting this finding.

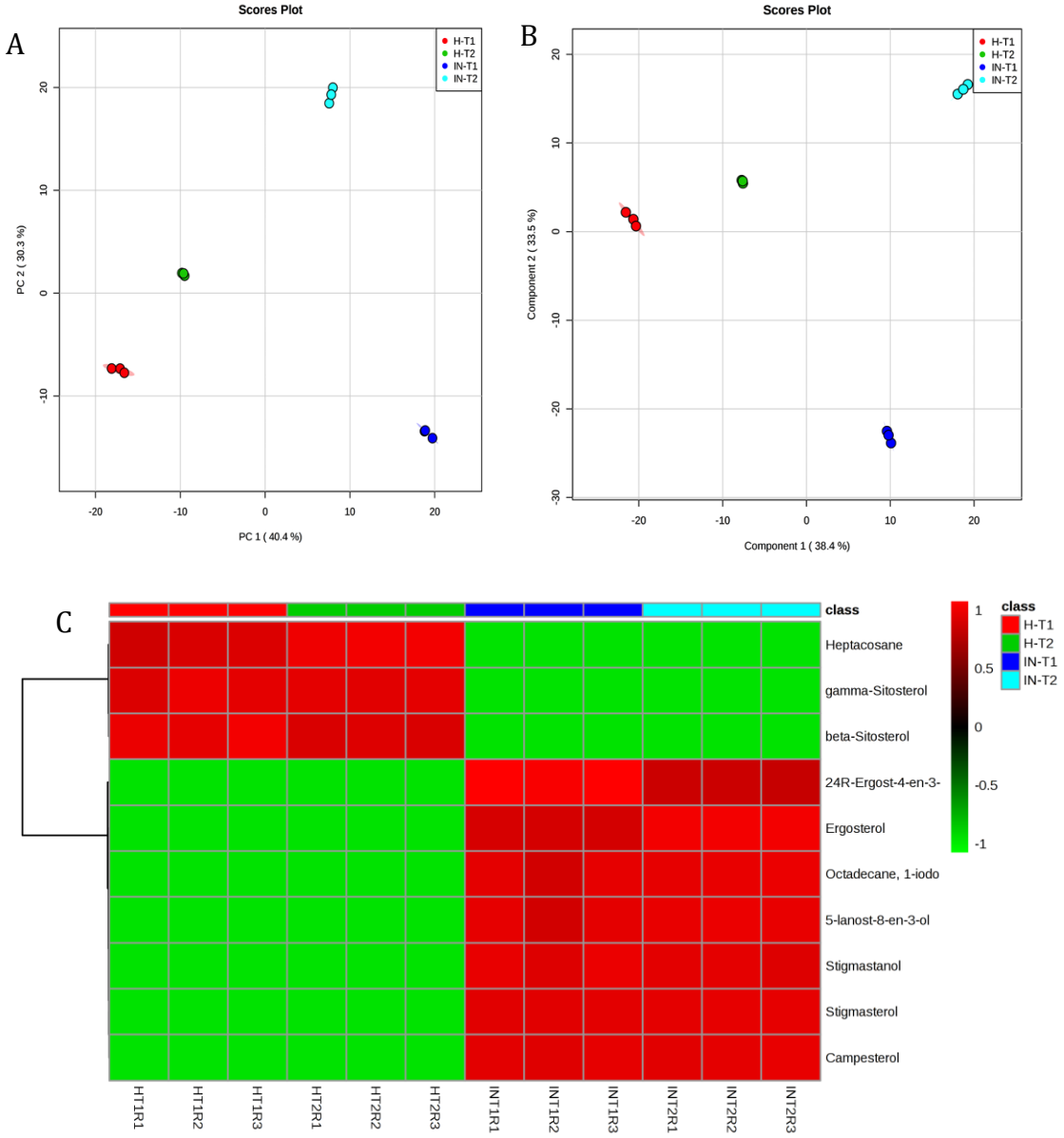

Figure 2. Multivariate cluster analysis of sterol profiles of H-T1 vs. IN-T1 vs. H-T2 vs. IN-T2 at first and second intervals. (A) Principal component analysis (PCA) score-plot. (B) Partial least square-discriminant analysis (PLS-DA) score-plot. (C) Heatmap visualization analysis of metabolites (VIP score > 1.5; p > 0.05) identified from PLS-DA model with a potential identity to distinguish between infected and healthy groups. Color key indicates metabolite expression value, green: lowest, red: highest. Analysis by Metaboanalyst consists n= 3 replicates for each groups. Notes: H=Healthy seedlings; IN=Ganoderma-inoculated seedlings; T1=First interval (3 months post-artificial inoculation (AI)); T2=Second interval (6 months post-AI).

Detection of fungal sterols, ergosterol only in infected oil palm seedling roots indicates the metabolites originating from *G. boninense* (Toh Choon *et al.,* 2011; Chong *et al.,* 2012). The molecule is a major sterol in fungi, perceived as a foreign molecule by plant cells thus triggering a series of defense responses against the intruder (Cervone *et al.,* 1999). Meanwhile, 5α-lanost-8-en-3β-ol, a triterpenoids reportedly found *G. applanatum* fruiting bodies extract thus explaining their detection in *Ganoderma*-infected palms (Shim *et al.,* 2004). Some sterol hydrocarbon chain is needed to accommodate within the lipid bilayer (Hodzic *et al.,* 2008), the detection of heptacosane and octadecane, 1-iodo- in healthy and infected oil palm.

IV. CONCLUSION

In conclusion, our results indicate that *Ganoderma* induced changes in the sterol composition on oil palm roots cell wall. Lipid sterols might play crucial role for molecular components/signaling recruitment in plant-defense responses. Accumulation of certain sterol compounds may also serves as important and relevant biomarkers in detecting *Ganoderma* infection at early stages.

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