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GC-MS Untargeted Comparative Metabolomics between Healthy and *Phytophthora palmivora* infected *Theobroma cacao* Pod Husk

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Abstract
Pod rot in *T. cacao* is overwhelming cacao orchards in the Philippines as the disease reduces the productivity of farms. Investigating the metabolite concentration of healthy and infected cacao pods to provide metabolomic information of the pathogenesis is the aim of this study. Metabolites extracted using *chloroform:methanol:water* solution of healthy and *P. palmivora* infected *T.cacao* were treated using untargeted GCMS. Ripe pods *T. cacao* were freshly collected from selected cacao orchard and were immediately preserved in liquid nitrogen before transported to the laboratory for further analyses. Analyte extraction was done following the protocols of metabolomic profile studies with some modifications. Data processing of the GC-MS results recorded the compounds suggested by the library with at least a similarity score of 70. The retention times of the compounds were manually aligned and their height were compared with the ribitol internal standard to obtain the relative quantification of the metabolites. The processed data were tabulated using MS Excel™ then subjected to Metaboloanalyst™ for PCA analysis. A total of 55 metabolites were observed in healthy pods while 59 for the infected pods. 28 of the combined metabolites were identified to be statistically different. These 28 metabolites are prime candidates for possible targeted metabolomic analysis or metabolomic markers for future studies on the pathogenesis and control of *P. palmivora* in cacao in the Philippines.

Keywords: cacao pod husk, comparative metabolomics, GC-MS, *Phytopthora palmivora, Theobroma cacao.*

1. Introduction

Theobroma cacao is a plant that is originally cultivated in Latin America and is now one of the main produces to some tropical countries in Africa and Asia along with its original source -the South America. The main product of the plant is the flavorful yet bitter seeds which is ultimately converted to chocolates that is well loved in the world. This product is one of the driving elements in economy of some developed countries particularly European countries that make the chocolates. Though chocolates are mostly manufactured in developed countries, the raw material – cacao, is produced in tropical countries such as the Philippines. Several areas in the Philippines cultivate cacao, however, the most prominent is Davao Region which was recently declared as the chocolate capital of the country. Cacao farmers in the region mostly prefer to cultivate the UF18 clone due to the significantly larger pods and denser beans compared to the other clones cultivated in the country.

The cultivation of *T. cacao* in developing countries has been going on for several years. Some cacao orchards, particularly in Africa, have been in the cacao industry for at least 100 years. Despite the long history on cacao production, cacao orchards are still not free from problems. The occurrence of pest and diseases in cacao is still one of the main sources of decrease in cacao production. These losses can reach of up to 30% (Puig *et al*, 2021, Delgadillo-Duran *et al*, 2020 and Iwaro *et al*, 1997). This reduction is clearly hampering the yield of farms and the chocolate industry as a whole. There are several pests and diseases that are encountered in any cacao orchards in the world. The moist condition of the Philippines is a great habitat for pathogens. Of the several pests and pathogens identified, the black pod or pod rot, caused by the oomycete, *Phythophthora pamilvora* is the most widespread in the country. The black pod disease is very distinctive in cacao as the pod changes its color to black or dark brown starting from a point or several points or area on the pod until the whole fruit is totally blackened. Furthermore, there is variability in the aggressiveness and pathogenicity of the oomycete (Masanto *et al*, 2021 and Surujdeo *et al*, 2016) which may have contributed to its spread in the Philippines.

Currently, there are several studies on black pod and *Theobroma cacao*. However, these studies are focused on the agricultural side and not as means of exploring the interrelation of the two. Furthermore, the mode of infection of the pathogen in the pod has never been extensively studied particularly the variants in the Philippines. Gas Chromatography with Mass Spectroscopy has been used in the study of cacao (Wang *et al*, 2016). These studies served as a template for the other MS analyses of cacao. These MS researches are vital as it can be applied to several studies and useful for further improvements of previous researches. As far as the authors are concerned, there is no study available at present comparing the GC-MS metabolite profile of a healthy and black pod infected cacao. Information from this metabolite profile may provide metabolomic marker essential for the initiation and termination of the pathogenesis as well as the metabolomic pathway of the metabolites responsible for the disease. Results from this investigation will pave the targeted analysis of the metabolites and its interaction that may lead to possible perpetual control of the pathogen in the Philippines.

2. Materials and Methods

Overview of the Analyses

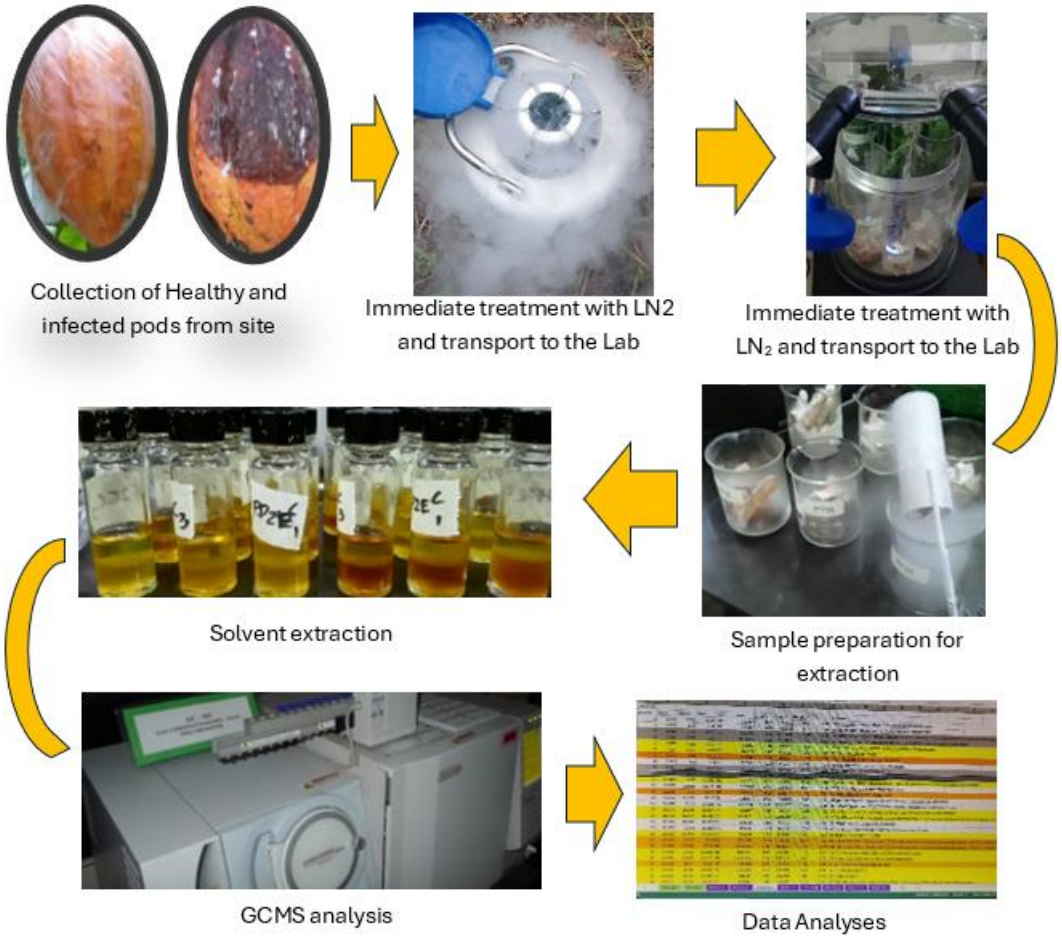

Figure 1. *Schematic diagram of the procedure used in the study. The study started with the identification and collection of samples from the sampling site followed by preservation. The samples were then transported to the lab for further processing. Then the samples were prepared for solvent extraction. The resultant extracted solvent was then subjected to GCMS analyses then data analyses.*

Collection of samples

The cacao analyses started with the collection of the samples. The sampling site and type of cacao pod husk were seriously considered for the study. Since majority of the cultivated cacaos in the Davao Region is UF18, the type of sample clone was delimited to UF18. Samples were only collected from the major cacao growing site in Davao which is Calinan to which Malagos is a part of it. This sampling strategy focused on the major cacao variant cultivated in the cacao dominated area of the region. Ripe healthy and black pod infected UF18 cacao pods husk were collected on site from a selected cacao orchard in Malagos, Davao City. Collection of the samples happened around 1000 to 1200 hours to establish optimum metabolomic activity in the plant samples. The selected pods were immediately cut into small sizes and submerged to a container with liquid nitrogen. This process halts any metabolomic activity preserving the metabolites and its concentration in the pod. The collected samples were immediately transported to the Chemistry Laboratory of Ateneo de Davao

University for further processing. The samples in liquid nitrogen were lyophilized for at least 72 hours to ensure the complete removal of water which may cause hydrolysis thus altering the metabolites concentration. The lyophilized sample were then ground using a laboratory grinder. The resultant cacao dry powder was then stored in a -80°C freezer to prevent any degradation until further use. .

Analyte extraction

Different extraction method will extract different metabolites. There is no universal extraction process that can extract all metabolites. For this study, the target metabolites extracted are polar to semi polar or those that can be extracted using the extraction solvent. Analysis of the metabolomic profile of cacao was based on the study of Gross *et al*, (2016) and Boas *et al* (2005) with some modifications. Around 200 mg of the cacao dried powder was weighed on an analytical balance and placed in a 10-mL glass container. The sample was added with 3.75 mL of freshly prepared chloroform:methanol (1:2) mixture and was vortexed vigorously. Then, 1.25 mL of chloroform was added and then vortexed. Then a 1.25 mL of distilled water was added and vortexed again. Then 2 mL of the bottom organic layer was pipetted out and transferred to a new glass container and was completely dried in vacuum oven at 35°C.

The dried substance was treated with 500µL of 10% (m/v) methanolic KOH solution. The mixture was then incubated at 85°C for 40 minutes. The resultant mixture was then treated with 250µL dH2O and 750 µL of heptane which was then vortexed well. The mixture was allowed to stand and have the layers separate. The organic upper phase (heptane layer) was pipetted out (500 µL) and transferred to a GCMS vial. 10 µL of the internal standard ribitol (1 mg/mL) was then added. The addition of the internal standard will validate the method and the instrument analysis that follows as the standard is detectable and measurable. This will also serve as a means of relative quantification of the other metabolites detected by means of ratio and proportion calculation. The organic solvent heptane was evaporated using a vacuum oven at 35°C until completely dry. The temperature was purposively lowered to 35°C as opposed to the recommended temperature from the study of Gross *et al*, (2016) and Boas *et al* (2005) to reduce the possibility of thermodegradation of the metabolites and preventing possible fire incident as the solvent being evaporated is high flammable. Then the GCMS vial was allowed to cool then 50 µL of the derivatizing agent N,O-Bis(trimethylsilyl)trifluoroacetamide **(BSTFA)** was added. The addition of the derivatizing agent allows the metabolites that normally do not volatilize and be detected using GC-MS become detectable. The mixture was incubated for 1 hour at 65°C. The final solution was diluted with ethyl acetate and subjected to GCMS.

GC-MS Operational parameters

GS-MS metabolites analysis is one of the preferred method for metabolomics analysis. The consistency and reliability of the instrument makes the metabolomic research results credible. For this study, GCMS-QP2010 Ultra by Shimadzu was the instrument used and Restek RTI-5MS (0.25µm x 0.25µm x 30m) was the column used for this study. The operational parameters for the GCMS were:

GC parameters

Initial Column Oven temperature	: 70.0°C
Injection temperature	: 300.0°C
Injection mode	: splitless
Pressure	: 100.0 kPa
Column flow rate	: 1.53 mL/min

GC program/column oven conditions

Initial temperature	: 70.0°C
Initial hold time	: 5 minutes
GC run rate	: 5.0°C/min
End hold time	: 5 minutes

MS parameters

Ion source temperature	: 250.0°C
Interface temperature	: 250.0°C
Start m/z	: 35
End m/z	: 850

These operational parameters were optimized after several trials using the recommended method and other method optimization to increase the number of metabolites determine and reduce the run time while ensuring the internal standard is identifiable and quantifiable. Technical replicates were used to ensure the credibility of the suggested metabolites while removing the background noise. Furthermore, blank analysis using the diluent – ethyl acetate was ran after the third technical replicate.

Metabolites analysis

The metabolites analysis was conducted using the software – GCMS Postrun analysis of the instrument. The Total Ion Chromatogram (TIC) of the analyses generated more than 200 peaks to which most have poor similarity score. To efficiently target only the notable chromatograms, those with substantial concentration, the top 150 TICs based on heights were selected. The similarity score of the suggested compounds were recorded and the suggested compound's mass spectra were compared and corrected to the most viable compound. The height of the chromatograph was used for quantification instead of the usual area as the chromatograph might not have completely separated to one another. As this study is an untargeted metabolomics, much of the emphasis is on the identification and relative quantification as opposed to absolute quantification provided by area analysis. The compounds with similarity score of 70 and above were selected as they have higher degree of correct identification. The unavailability of standards for confirmation limits the accurate identification of the suggested compounds. Nevertheless, the high similarity score is a logical start for future confirmatory studies. The identified metabolites were then clustered and their relative concentrations were determined. The concentration of the metabolites in both the healthy and infected pods were compared. For efficient metabolomic analysis, only the metabolites with coefficient of variance (CV) using MS

Excel of less than 30% were considered. Then, student T test of the MS Excel was used to evaluate if the metabolite concentration changes in the healthy and infected pods were significant at 95% confidence level.

PCA analyses

Principal component analysis (PCA) was also used to the samples. The purpose of this test is to evaluate if the 2 sample are indeed different. This will also establish to what extent is the difference between the samples. Using the relative concentration of the metabolites, these values were encoded in the MS Excel and the resultant file was process by Metaboloanalyst™.

3. Results

PCA results

The result of the PCA analysis is shown **Figure 2**. From the illustration, majority of the trial results, regardless if the sample is from healthy or infected pod husk, are close to one another indicating less variation. Nevertheless, the two samples are still distinctly unique to one another as they are not overlapping. The current number of samples which is 12 for healthy and infected, even with its technical replicates, is still lacking. The delimitation of using infected pods with strict conditions of being ripe, infected up to the middle of the pod and located from Malagos and UF18 clone restricts most the possible sample candidates. Perhaps more test runs may prove to be different as the areas of the two groups suggest possible overlapping. Finally, the PCA result clearly shows an overall metabolomic difference between the healthy cacao pods against the *P. palmivora* infected cacao. This difference might be subtle for bulk of the samples, this suggests that the slight changes in the metabolomic concentration leads to the eventual pathogenesis of the oomycete. Admittedly, this statement should be validated with further studies such as targeted metabolomics amongst others.

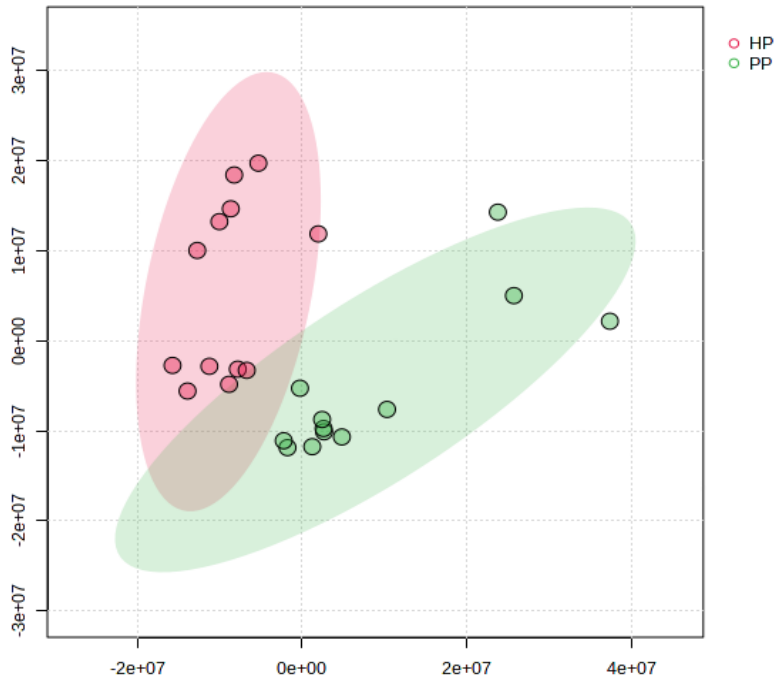

***Figure 2**. PCA result of the healthy (HP) vs P. palmivora infected cacao (PP) from metaboloanalyst. The circular dots represent healthy pods while the triangular are for the black pod infected pods. Most of the substances are in close proximity with one another as indicated by the box.*

Metabolites identified

Table 1 – List of Metabolites identified in the GCMS library with at least 70 similarity. The silylated compounds identified were converted to their non-silyl form. ϕAre compounds without significant difference in their concentration for both healthy and infected. ♦Are compounds with higher concentration in the healthy pods. σAre compounds with higher concentration in the infected. ♥Are only found in healthy pods. δAre only found in infected pods.

Compound Name	Formula	Classification
2,4,6-Trimethylpyridine$^{\phi}$	$C_8H_{11}N$	Alkaloid
Phosphoric acid$^{\phi}$	H_3PO_4	Acid
methyl hexadecanoate$^{\phi}$	$C_{17}H_{34}O_2$	FAME
Methyl heptadecanoate$^{\blacklozenge}$	$C_{18}H_{36}O_2$	FAME
Methyl linoleate$^{\blacklozenge}$	$C_{19}H_{34}O_2$	FAME
Methyl elaidate$^{\blacklozenge}$	$C_{19}H_{36}O_2$	FAME
methyl octadec-11-enoate$^{\blacklozenge}$	$C_{19}H_{36}O_2$	FAME
methyl 9,10-epoxystearate$^{\sigma}$	$C_{19}H_{36}O_3$	FAME
Methyl 10-oxooctadecanoate$^{\phi}$	$C_{19}H_{36}O_3$	FAME
methyl octadecanoate$^{\phi}$	$C_{19}H_{38}O_2$	FAME
methyl 9,10-dihydroxyoctadecanoate$^{\phi}$	$C_{19}H_{38}O_4$	FAME
Methyl 18-methylnonadecanoate$^{\phi}$	$C_{21}H_{42}O_2$	FAME
Methyl 8-[2-[[2-[(2-ethylcyclopropyl)methyl] cyclopropyl]methyl]cyclopropyl]octanoate$^{\sigma}$	$C_{22}H_{38}O_2$	FAME
methyl (8E,11E,14E)-8,11,14-docosatrienoate$^{\delta}$	$C_{23}H_{40}O_2$	FAME
methyl docosanoate$^{\phi}$	$C_{23}H_{46}O_2$	FAME
Tetradecanoic acid$^{\phi}$	$C_{14}H_{28}O_2$	Fatty acid
n-Pentadecanoic acid$^{\sigma}$	$C_{15}H_{30}O_2$	Fatty acid
cis-9-Hexadecenoic acid$^{\delta}$	$C_{16}H_{30}O_2$	Fatty acid
Hexadecanoic acid$^{\sigma}$	$C_{16}H_{32}O_2$	Fatty acid
Linoleic acid$^{\sigma}$	$C_{18}H_{32}O_2$	Fatty acid
trans 9-octadecenoic acid$^{\phi}$	$C_{18}H_{34}O_2$	Fatty acid
Octadecanoic acid$^{\sigma}$	$C_{18}H_{36}O_2$	Fatty acid
Eicosanoic acid$^{\sigma}$	$C_{20}H_{40}O_2$	Fatty acid
Heneicosanoic acid$^{\phi}$	$C_{21}H_{42}O_2$	Fatty acid
Docosanoic acid$^{\phi}$	$C_{22}H_{44}O_2$	Fatty acid
Tetracosanoic acid$^{\phi}$	$C_{24}H_{48}O_2$	Fatty acid
Pentacosanoic acid$^{\phi}$	$C_{25}H_{50}O_2$	Fatty acid
Hexacosanoic acid$^{\delta}$	$C_{26}H_{52}O_2$	Fatty acid
2-hydroxyhexacosanoic acid$^{\delta}$	$C_{26}H_{52}O_3$	Fatty acid
Bis(2-ethylhexyl) decanedioate$^{\phi}$	$C_{26}H_{50}O_4$	Fatty acid ester
9,12-Octadecadienoyl chloride$^{\phi}$	$C_{18}H_{31}ClO$	Fatty acid halide
Docosyl trifluoroacetate$^{\delta}$	$C_{14}H_{25}F_3O_2$	Fatty acid halide
6-Undecanol$^{\phi}$	$C_{11}H_{24}O$	Fatty alcohol

1-Tetradecanol♥	$C_{14}H_{30}O$	Fatty alcohol
1-Hexadecanol♦	$C_{16}H_{34}O$	Fatty alcohol
n-heptadecanol♦	$C_{17}H_{36}O$	Fatty alcohol
1-octadecanol♦	$C_{18}H_{38}O$	Fatty alcohol
1-tetracosanol♦	$C_{24}H_{50}O$	Fatty alcohol
1-hexacosanol♠	$C_{26}H_{54}O$	Fatty alcohol
n-Decane♦	$C_{10}H_{22}$	Hydrocarbon
1-Octadecene♣	$C_{18}H_{36}$	Hydrocarbon
heneicosane♠	$C_{21}H_{44}$	Hydrocarbon
Pentacosane♦	$C_{25}H_{52}$	Hydrocarbon
n-Dotriacontane♥	$C_{32}H_{66}$	Hydrocarbon
Tetracontane♥	$C_{40}H_{82}$	Hydrocarbon
Lactic acid♦	$C_3H_6O_3$	Organic acid
Glycolic acid♦	$C_2H_4O_3$	Organic acid
dibutylhydroxytoluene♠	$C_{15}H_{24}O$	Phenolic
Butyl methyl phthalate♦	$C_{13}H_{16}O_4$	Phthalates
Dibutyl phthalate♠	$C_{16}H_{22}O_4$	Phthalates
1,3 propanediol♦	$C_3H_8O_2$	Polyol
glycerol♦	$C_3H_8O_3$	Polyol
Ergosterol♥	$C_{28}H_{44}O$	Sterol
2-Ketogluconic acid♥	$C_6H_{10}O_7$	Sugar acid
erythritol♦	$C_4H_{10}O_4$	Sugar alcohol
6,10,14-Trimethyl-2-pentadecanone♦	$C_{18}H_{36}O$	Terpene
4,8,12,16-Tetramethylheptadecan-4-olide♦	$C_{21}H_{40}O_2$	Terpene
Squalene♦	$C_{30}H_{50}$	Terpene
Farnesol♠	$C_{15}H_{26}O$	Terpene alcohol
phytol♦	$C_{20}H_{40}O$	Terpene alcohol
Phytanol♦	$C_{20}H_{42}O$	Terpene alcohol
Pantothenic acid♥	$C_9H_{17}NO_5$	Vitamin
alpha-Tocopherol♣	$C_{29}H_{50}O_2$	Vitamin

FAME – fatty acid methyl ester

Discussion

This study was able to identify 55 metabolites (51 common and 4 unique) from healthy cacao pod husk while 59 (51 common and 8 unique) metabolites were identified from *P. palmivora* infected cacao pods. This difference suggests that the some metabolites may have been generated as a response to the plant-pathogen interaction. This finding needs to be validated using targeted metabolomics or other omics science. Furthermore, 28 common metabolites from the two samples had significant difference in their relative concentrations. These metabolites or changes in metabolite concentration might have been affected by action or interaction of the pathogen with the pod husks.

Most of the compounds identified were methyl esters, fatty acids, hydrocarbons, fatty alcohols and terpenes. Similar to the study of Rachmawaty *et al* (2018) they were able to identify the presence of some fatty acid methyl esters to which most where also suggested in this study. In contrast with the study of Gallego *et al* (2022) were they analyzed the metabolomic difference of a ripening cacao pod husks, none of the results here are similar to what they had. Perhaps the difference in instrument used for the analyses was key in the difference. Furthermore, a similar study by Meza-Sepulveda et al (2024) on cacao clones from Colombia suggested the presence of sugars, reducing sugars and phosphorus. However, because they did not utilize GCMS, the results were hardly comparable with the GCMS results of this study.

Interestingly, an alkaloid was also found in this study which was not consistent with other studies indicating a higher concentration for alkaloids (Yahya *et al*, 2021 and Barrios-Rodriguez *et al*, 2022). Furthermore, polyphenols or the phenolics was found to be limited in this study. The study of Vriesmann *et al* (2011) suggests that the contents and amount of polyphenols in cacao beans varies and that the concentration in husk is very limited. Perhaps, most of the polyphenols are in the beans and not in the husk as these antioxidant polyphenols are much needed by the beans for its development rather than the pod husk. Interestingly, the results indicate that the presence of theobromine and caffeine – which are the dominant metabolites in beans (Belwal, 2022) are absent. Despite running the experiment with a theobromine and caffeine standard, the chromatogram results did not show them. Perhaps their concentration was too low for it to be detected with higher accuracy.

The effect of *P. palmivora* to the pod husk's metabolites were also observed in the study. Most of these metabolites are secondary metabolites which may provide protection against pathogens or may function to help plants communicate with the microorganism (Anjali *et al*, 2023). These changes might have been due to the formation of pathogen resistance action of the plant (Perrine-Walker, 2020) as a form of post-penetration resistance given that the test samples are between healthy and currently infected pods. The metabolites with differentiated concentrations are the following:

Fatty acid methyl esters (FAME)
These fatty acid derivatives are good indicators of the pods health. Methyl esters of C18 and C20 indicates healthy pods and must have been a key factor as to its resistance. Furthermore, the formation of epoxy group and cyclopropane ring in the tail of the fatty acid which are sterically unfavorable compounds were manifested in the infected husks. These suggests that the trienenoate which can also be found in the infected plants must have reacted to the pathogenic metabolites forming the sterically unfavorable compounds. It has been observed that unsaturated fatty acids have various role in pathogenesis (Mei, 2020), these may not be directly reacting to the pathogens but may be in the form of signal for other defense mechanism pathway.

Fatty acids (FA)
Most of the identified FA had lower concentration in the *P. palmivora* infected husk. These downregulation of the FAs might be attributed to the defense response of the plant. The formation of cis-9-hexadecenoic acid in the infected husks suggests the establishment of a defense mechanism according to Lim *et al* (2017) to which the said compound is converted to azelaic acid to combat the pathogen. Moreover, the formation of very long chain fatty acids (VLCFA) such as the hexacosanoic acid and 2-hydroxyhexacosanoic acid in the infected pods supports the study of Batsale *et al* (2021) to which the formation of these VLCFA as a form of adaptation of the plants during biotic and abiotic stress.

Terpenes – farnesol
Several terpenes and derivatives were identified in this study. In the study of Divekar *et al* (2022), it suggests that terpenes among others, has demonstrated to serve as potential plant defense regulators. In particular, the higher concentration of farnesol in healthy pods is interesting. For one, elevated concentration of farnesol is toxic to the plants (Cardoza *et al*, 2022). However, the same study relates farnesol to squalene and ergosterol differentiated concentration when a certain pathogen was introduced. This highlights the possibility of farnesol's decreased concentration in the infected husk as due to the pathogen response activity.

Phenolic – dibutylhydroxytoluene (BHT)
The study of Yan *et al*, (2021) suggests that BHT is related to the defensive enzymes production. Moreover, the production of salicylic acid was enhanced, to which this compound has been associated with plant defense. The reduced concentration of BHT in the infected husk must have consumed the BHT to produce more defense enzymes and salicylic acid. Unfortunately, the method was unsuccessful in quantifying salicylic acid. However, the formation of ergosterol in the infected husks was observed supporting the study of Yan *et al* (2021).

Vitamin – pantothenic acid (B vitamin)
The study of Hanson *et al* (2016) suggested that B vitamins are the precursors of important metabolic cofactors, however, they are prone to destructions under stress conditions. This might explain as to why the presence of pantothenic acid was absent in the infected plants.

Hydrocarbons
The hydrocarbons identified in this study have different quantities in both healthy and infected husks. Though some hydrocarbons such as the tridecane and hexadecane have been reported to support plant defense by promoting the formation of salicylic acid (Ramirez *et al*, 2020). Most of these long chain hydrocarbons exist in plants as a form of wax. These wax prevents the evaporation of water as well as protection against phytopathogens (Kant et al, 2015). These hydrocarbons need to be explored further as their presence and concentrations vary in healthy and infected husks.

4. Conclusions

The metabolomic difference between the healthy and *P. palmivora* infected cacao pod husks were investigated in this study using GC-MS. The results improved the number of metabolites previously identified in cacao pod husks using GCMS. 51 common metabolites were observed in both healthy and infected cacao pods to which 4 are unique to healthy pods and 8 from the infected pods. The higher number of metabolites in the infected pods suggest the formation of new metabolites stemming from the plant-pathogen interaction which may have affected the formation of unique metabolites from healthy pods. Furthermore, 28 common metabolites had their concentrations significantly altered due to the pathogenesis. The formation, deletion, upregulation and downregulation of some metabolites suggest a dynamic response by the host plant to combat the presence of the pathogen. The current study provides significant information in the quest to combat pathogenesis by P. palmivora. Ironically, the same result puts more questions as to why some metabolites concentration changed. The challenge to explore this question is severely reliant on the expansion of this study by using targeted approach, increased number of test samples, other omics approach and using other tools such as LCMS/MS or NMR. This research will do well if paired with proteomic investigations to look into the different proteins generated and its interaction with the pathogen.

5. References

Anjali, S., Korra, T., Thakur, R., Arutselvan, R., Kashyap, A.S., Nehela, Y., Chaplygin, V., Minkina, T., and Keswani, C.(2023) Role of plant secondary metabolites in defence and transcriptional regulation in response to biotic stress. Plant Stress. vol 8, 100154. doi.org/10.1016/j.stress.2023.100154.

Barrios-Rodríguez, Y.F., Salas-Calderón, K.T., Orozco-Blanco, D.A., Gentile, P. and Girón-Hernández, J. (2022), Cocoa Pod Husk: A High-Pectin Source with Applications in the Food and Biomedical Fields. CBEN, 9: 462-474. https://doi.org/10.1002/cben.202100061

Batsale, M., Bahammou, D.,Fouillen, L.,Mongrand, S., Joubès, J., and Domergue, F.(2021). Biosynthesis and Functions of Very-Long-Chain Fatty Acids in the Responses of Plants to Abiotic and Biotic Stresses. Cells 10, no. 6: 1284. https://doi.org/10.3390/cells10061284

Belwal, T., Cravotto, C., Ramola, S., Thakur, M., Chemat, F., and Cravotto, G. (2022). Bioactive Compounds from Cocoa Husk: Extraction, Analysis and Applications in Food Production Chain. Foods 11, no. 6: 798. https://doi.org/10.3390/foods11060798

Boas, S. et al. (2005) Global metabolite analysis of yeast. Evaluation and sample preparation methods. Yeast. 22, 1155-1169

Cardoza, R., McCormick, S., Lindo, L., Mayo-Prieto, S., González-Cazón, D., Martínez-Reyes, N., Carro-Huerga, G., Rodríguez-González, A., Proctor, R., and Casquero, P. (2022). Effect of Farnesol in Trichoderma Physiology and in Fungal–Plant Interaction. Journal of Fungi 8, no. 12: 1266. https://doi.org/10.3390/jof8121266

Dedyukhina, E.G., Kamzolova, S.V. & Vainshtein, M.B. (2014). Arachidonic acid as an elicitor of the plant defense response to phytopathogens. Chem. Biol. Technol. Agric. 1, 18. https://doi.org/10.1186/s40538-014-0018-9

Delgadillo-Durán, P., Soto-Suárez, M., Rodriguez-Polanco, L. et al. (2020) A new method for the inoculation of Phytophthora palmivora (Butler) into cacao seedlings under greenhouse conditions. Plant Methods 16, 114. https://doi.org/10.1186/s13007-020-00656-8

Dewi, V.S. et al (2020). Resistance test of five cocoa (Theobromae cacao L.) clones in South Sulawesi against Phytophthora palmivora. IOP Conf. Ser.: Earth Environ. Sci. 486 012173

Divekar PA, Narayana S, Divekar BA, Kumar R, Gadratagi BG, Ray A, Singh AK, Rani V, Singh V, Singh AK, Kumar A, Singh RP, Meena RS, Behera TK. (2022). Plant Secondary Metabolites as Defense Tools against Herbivores for Sustainable Crop Protection. Int J Mol Sci. 28;23(5):2690. doi: 10.3390/ijms23052690. PMID: 35269836; PMCID: PMC8910576.

Gallego, A., Zambrano, R., Zuluaga, M., Camargo-Rodriguez, A., Candamil-Cortes, M., Vergel, A., Valencia, J. (2022) Analysis of fruit ripening in Theobroma cacao pod husk based on untargeted metabolomics, Phytochemistry. Volume 203, 113412. https://doi.org/10.1016/j.phytochem.2022.113412.

Hanson, A. D., Beaudoin, G. A., McCarty, D. R., & Gregory, J. F., 3rd (2016). Does Abiotic Stress Cause Functional B Vitamin Deficiency in Plants? Plant physiology, 172(4), 2082–2097. https://doi.org/10.1104/pp.16.01371

He, M. and Ding, N. (2020). Plant Unsaturated Fatty Acids: Multiple Roles in Stress Response. Frontiers in Plant Science. Vol 12. DOI:10.3389/fpls.2020.562785

Huang, Y., Sun, C., Guan, X, Lian., S, Li, B., Wang, C. (2021). Butylated Hydroxytoluene Induced Resistance Against Botryosphaeria dothidea in Apple Fruit. Frontiers in Microbiology. Vol 11. DOI:10.3389/fmicb.2020.599062

Iwaro, A. D., Sreenivasan, T. N., and Umaharan, P. (1997). Foliar resistance to Phytophthora palmivora as an indicator of pod resistance in Theobroma cacao. Plant Dis. 81:619-624.

Kant, M.R., Jonckheere,W., Knegt,B., Lemos, F., Liu, J., Schimmel, B.C.J., Villarroel, C.A., Ataide, L.M.S., Dermauw, W., Glas, J.J., Egas, M., Janssen, A., Van Leeuwen,T., Schuurink, R.C., Sabelis, M.W., and Alba, J.M.(2015) Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. Annals of Botany, Vol 115, Issue 7, June 2015, pp 1015–1051, https://doi.org/10.1093/aob/mcv054

Lim, G.-H., Singhal, R., Kachroo, A., & Kachroo, P. (2017). Fatty Acid– and Lipid-Mediated Signaling in Plant Defense. Annual Review of Phytopathology, 55(1), 505–536. doi:10.1146/annurev-phyto-080516-035406

Masanto, M., Wibowo, A., Ridwan, N. F., Sawitri, W. D., Kageyama, K., & Subandiyah, S. (2021). The expression of pathogenicity-related genes in Phytophthora palmivora causing black pod rot disease on cacao (Theobroma cacao L.) in Indonesia. Journal of Plant Interactions, 16(1), 284–295. https://doi.org/10.1080/17429145.2021.1942571

Mayorga-Gross, A.L. et al. (2016) An untargeted metabolomic assessment of cacao beans during fermentation. Food research International. 89. Pp. 901-909

Meza-Sepulveda DC, Hernandez-Urrea C, Quintero-Saavedra JI. (2024) Physicochemical characterization of the pod husk of Theobroma cacao L. of clones CCN51, FEAR5, and FSV41 and its agroindustrial application. Heliyon. 2024 Apr 4; 10(7), e28761. doi: 10.1016/j.heliyon.2024.e28761. PMID: 38601635; PMCID: PMC11004208.

Perrine-Walker F. (2020). Phytophthora palmivora-Cocoa Interaction. Journal of fungi, 6(3), 167. https://doi.org/10.3390/jof6030167

Puig, A. S., Quintanilla, W., Matsumoto, T., Keith, L., Gutierrez, O.A., and Marelli, J. (2021) Phytophthora palmivora Causing Disease on Theobroma cacao in Hawaii. Agriculture 11, no. 5: 396. https://doi.org/10.3390/agriculture11050396

Rachmawaty, A., et al (2018). Active Compounds Extraction of Cocoa Pod Husk (Thebroma Cacao l.) and Potential as Fungicides J. Phys.: Conf. Ser. 1028 012013,

Ramírez V, Munive J-A, Cortes L, Muñoz-Rojas J, Portillo R and Baez A (2020) Long-Chain Hydrocarbons (C21, C24, and C31) Released by Bacillus sp. MH778713 Break

Dormancy of Mesquite Seeds Subjected to Chromium Stress. Front. Microbiol. 11:741. doi: 10.3389/fmicb.2020.00741

Ramírez, V., Munive, J. , Cortes, L. , Muñoz-Rojas, J., , Portillo, R.,and Baez, A. (2020) Long-Chain Hydrocarbons (C21, C24, and C31) Released by Bacillus sp. MH778713 Break Dormancy of Mesquite Seeds Subjected to Chromium Stress. Frontiers in Microbiology. vol 11. DOI:10.3389/fmicb.2020.00741

Ramírez-Gómez, X., Jiménez-García, S., Campos, V.B., and Campos, M. (2020) Plant Metabolites in Plant Defense Against Pathogens. Plant Diseases - Current Threats and Management Trends. IntechOpen. doi:10.5772/intechopen.87958.

Surujdeo-Maharaj, Surendra & Sreenivasan, T. & Motilal, Lambert & Umaharan, Path. (2016). Black Pod and Other Phytophthora Induced Diseases of Cacao: History, Biology, and Control. 10.1007/978-3-319-24789-2_7.

Vriesmann, L. et al (2011). Cacao pod husks (Theobroma cacao L.): Composition and hot-water-soluble pectins. Industrial Crops and Products - IND CROPS PRODUCTS. 34. 1173-1181. 10.1016/j.indcrop.2011.04.004.

Wang, H. et al (2016). System level analysis of cacao seed ripening reveals a sequential interplay of primary and secondary metabolism leading to polyphenol accumulation and preparation of stress resistance. The plant journal. 87, 318-332

Yahya M, Ginting B, Saidi N. (2021) In-Vitro Screenings for Biological and Antioxidant Activities of Water Extract from Theobroma cacao L. Pod Husk: Potential Utilization in Foods. Molecules. 16;26(22):6915. doi: 10.3390/molecules26226915.