Identification of metabolite compounds on Robusta Coffee (*Coffea canephora*) roots related to parasitic nematodes of *Prathylenchus coffeae*

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Abstract. The *Prathylenchus coffeae* is a significant plant-parasitic nematodes in *Coffea canephora*. This study examines the identification of metabolite compounds related to plant defense against parasitic nematodes by extracting *C. canephora* roots with n-Hexane and Gas Chromatography-Mass Spectrometry analysis of the prepared extract. Resistant coffee varieties (BP 308) and susceptible coffee varieties (BP42, BP 409, and BP 358) were used in this study. GC-MS's data indicates the presence of forty-seven, thirty-nine, fifty, and thirty metabolite compounds in roots of BP 308, BP 42, BP 409, and BP 358, respectively. The principal component analysis (PCA) analysis using the loading plot model was conducted for reliable and accurate discrimination to identify potential metabolite compounds that serve as marker compounds and can distinguish between nematode-resistant and susceptible varieties. The results showed that each coffee variety has different metabolite compound characteristics. BP 409 was characterized by Octadecanal and Tetradecanal, BP 308 by Phenol and Guaiene and BP 358 and BP 42 by Cholest and Patchouli. This study confirmed candidate metabolite markers that differentiate coffee varieties resistant to nematode. Furthermore, the data presented may help develop a new method for detecting resistant and susceptible coffee against nematode attacks caused by *Prathylenchus coffeae*.

1 Introduction

PPNs (plant-parasitic nematodes) are the greatest destructive pests of crops globally, causing an estimated $80 billion in production losses each year [1]. Although nematode infection is rarely deadly, PPN reduces crops dramatically by stealing nutrients, affecting water movement, enhancing sensitivity to subsequent infections, and serving as a vehicle for viruses [2]. Coffee-PPN *Pratylenchus coffeae* triggers numerous degradation of coffee root cell membranes, decreasing water and nutrient absorption, the photosynthesis process, and carbohydrates downward delivery; these mechanisms cause indicators observed in parasitized coffee plants, such as stunning, major chlorosis, and leaf shattering. The attack of

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nematode *P. coffeae* was reported to have caused the extinction of 95% of coffee plantations in Java, Indonesia [3]. The amphimitic *P. coffeae* is highly habituated to tropical climates in coffee plantations. These species cannot survive in temperatures of the soil below 10 ˚C or above 32 ˚C, as well as with a humidity level below 2%. Their lifespan in complying with land is not exceeding four months, however they can persist for no less than nine months in decomposing roots; different habitats are likewise essential for these organisms' pathogenesis. Edaphic environments are not expected to have influenced the spread of *Pratylenchus* spp. in coffee.

Many strategies can be employed to prevent nematode attacks, but according to [5] adopting a resistant variety, technical cultivation methods and biological agents are suitable components for integrated nematode/pest control [6]. Pest control efforts are directed to environmentally friendly control [3], which is the most successful technique for nematode handling, with lower costs and fewer environmental residues. Eloh *et al*. utilized gas chromatography-mass spectrometry (GC-MS) to discover metabolite characteristics of nematode associations with tomato cultivars before and following inoculation [7, 8]. Afifah *et al*. investigate the metabolites strategy for determining the tolerance of four tomato species (*Solanum lycopersicum* L.) against root-knot worms (*Meloidogyne incognita*) [9, 10].

However, only certain research has employed metabolites to investigate the interconnections of coffee cultivation and parasitic nematodes. Metabolic examined in the roots of coffee cultivation subjected to the coffee root-knot nematode, *Meloidogyne exigua* has been comprehensively discussed by Machado et al. [11]. The study of defense mechanisms against PPNs *P. coffeae* is comparatively less than Root-Knot Nematode. However, there have not been extensive studies of metabolite profiles to investigate the associations among coffee cultivars that are potentially vulnerable or immune against infected parasitic nematodes of PPNs, so the defense mechanism by the metabolite approach remains largely undiscovered. In Indonesia, there is not sufficient data available about plant-parasitic nematode species that influence coffee. This is essential because different metabolite profiles can indicate alterations in metabolic mechanisms, allowing normal and disease environments in an organism to become detached [12]. Analyzing the metabolite compositions of resistant and susceptible populations is an effective method for determining metabolic processes implicated in multifaceted disorders. Plants synthesize many different substances with varying compositions [13]. Non-targeted metabolites have been utilized to better understand the homeostatic biochemistry process of quantifying tolerance in agricultural crops to various of diseases [14]. Metabolite identification allows researchers to draw decisions regarding the response strategies employed by economically significant plants [15]. On the other hand, because of the unique features of metabolites, a repository of common chemicals is required to serve as a foundation for data evaluation. There is currently no comprehensive statistical evaluation platform for coffee in Indonesia; therefore, another investigation must be performed to establish a foundation for future research.

The present research performed an initial separation approach accompanied by a GC-MS method to explore the compounds related to resistance and susceptibility of four different coffee varieties (BP 308, BP 49, BP 42, and BP 356). The determination of compounds that trigger alterations among resistant and susceptible cultivars may bring insight into the biochemical variability of transmitting compounds that contribute to plant-parasitic nematode (PPNs) attacks. The present investigation aimed at analyzing variations in metabolite characterization of coffee cultivars between resistance and susceptible, that were in charge of conferring defense against *P. coffeae* in tolerance varieties.

2 Material and methods

2.1 Nematode inoculum

The aggregation of *P. coffeae* nematode isolates was carried out in an area endemic to nematode attack at the Kaliwining Experimental Field, Indonesian Coffee and Cocoa Research Institute, Jember, East Java. The reproduction and extraction were carried out at the Nematology Laboratory, Indonesian Coffee and Cocoa Research Institute, Jember, East Java based on the modified Baermann protocol [16].

2.2 Inoculation of coffee seedlings

Coffee seedlings consist of four robusta coffee varieties from cuttings propagation, including resistant (BP 308) and susceptible (BP 409, BP 42, and BP 358) clones. Each treatment consisted of 5 seeds inoculated with 100 nematodes per polybag and 5 seeds not inoculated (as control). Therefore each variety needed as many as 10 seeds from cuttings. Nematode inoculation was carried out after the coffee seedlings were 14 days old.

2.3 Root extraction

Roots were collected from seedling coffee in the greenhouse of the Indonesian Coffee and Cocoa Research Institute. The roots were air-dried after being cleaned with faucet water. The dehydrated specimens were broken up into little fragments and blended into a fine flour. The maceration of the crushed coffee root in n-hexane was completed while it was agitated for almost an hour. The resulting substance was then inoculated for a full day and subsequently purified three times. Viscose extract was obtained by distilling the residue at 50˚C under a rotary evaporator pressure until the oil and solvent were divided.

2.4 Quantification of metabolite compounds in the roots of coffee plants

Identification of metabolite compounds of coffee root extract was determined using Gas Chromatography-Mass Spectrometry (GC-MS QP2010 Plus) Shimadzu using capillary column RTx5MS (cross bond 5% diphenyl-95% dimethylpolysiloxane) with 30m x 0.25mm x 0.25 μm film thickness. A sample of 1 μl was injected into GC-MS with an oven temperature of 80-250 ˚C with a pressure of 60 kPa, a total rate of 6.0 ml/minute, a column rate of 0.94 ml/minute, linear velocity of 35.7 cm/second, Purge Flow 3.0 ml/minute and a split ratio of -10.

2.5 Statistical analysis

Using the RStudio software, principal component analysis (PCA) and biplot analysis were applied to identify the critical biochemical indicators that can distinguish among cultivated plant immunity categories.

3 Results and discussion

Actually a novel investigation to examine the variance in the chemical makeup of robusta coffee varieties that are immune yet vulnerable to nematode attacks induced by *P. coffeae.* The composition of metabolic products in plants infected 14 weeks after inoculation was

fully demonstrated by the unintended compounds procedure utilized in this study, indicating a description of the crop's persistent resistance. Breeding initiatives can be supported by using a metabolites strategy. Plant breeders can uncover chemicals that are crucial for the resistance of plants against diseases and pests through this method. Numerous physiologically active compounds and additional chemicals are generated by tolerant plants that increase their resistance against pests and diseases. The study of metabolic function is an effective technique for assessing how crops respond against exposure to nematodes [9]. Numerous plants have been subjected to metabolomics analysis in association with nematode tolerance for example *Solanum Lycopersicum* L. [8, 9 17], *Psidium guajava* [18], *Glycine max* (L.) [19, 20].

GC-MS was used to identify the components that were extracted from coffee roots (Fig. 1). Four various kinds of coffee root samples produced various complete element chromatograms. The four coffee species' average element present diagrams show that there are differences in the total amount of peaks and intensities of each variety. A wealth of facts and figures are available from this research to assist in describing compounds. By monitoring the compounds' output cycle quantity, variations in compounds were discovered. Fig. 2, 3, 4 and 5 presented a typical GC-MS chromatogram of n-hexane fraction from BP 42, BP 308, BP 358, and BP 409 coffee varieties. There were thirty-nine metabolites detected in BP 42, while forty-seven metabolites were detected in BP 308. Thirty metabolites were detected in BP 358 samples, while fifty metabolites were detected in BP 409 samples.

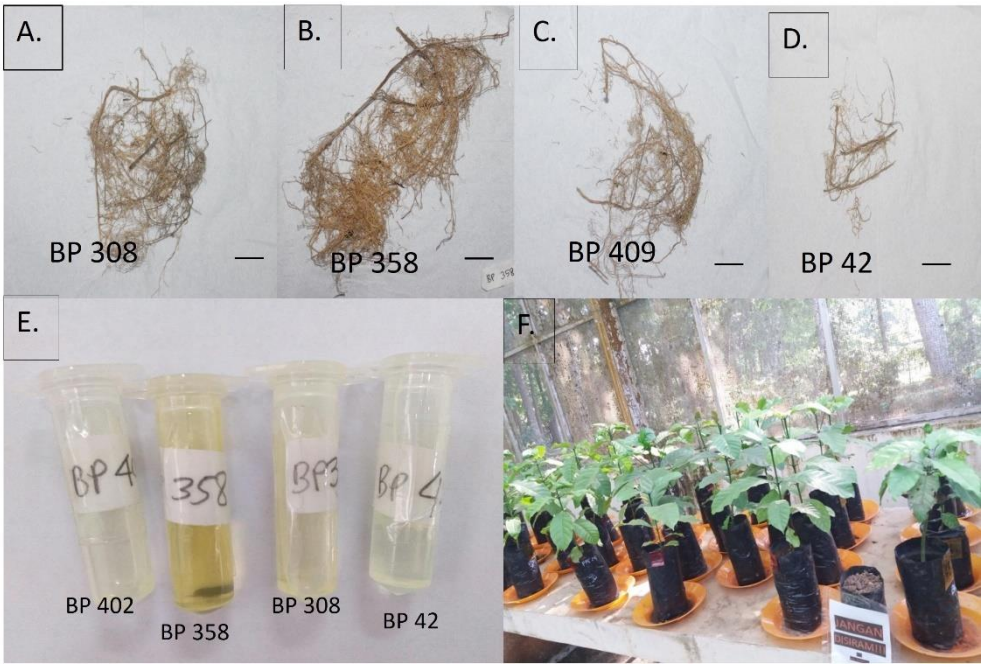

Fig. 1. Samples of dried coffee roots of BP 308 (A), BP 358 (B), BP 409 (C), and BP 42 (D). The fraction of n-hexane from coffee roots (E). Coffee samples were infected nematodes *Pratylenchus coffeae* (F).

The n-hexane extract's initial chemical compound evaluation revealed an abundance of amino acids, proteins, carbohydrates, fixed oil, steroids, flavonoids, alkaloids, and phenol in abundance as the major constituents (Table 1). An overall number of 166 signals were discovered during the GC-MS compound screening; however, due to their superior

chromatogram, only 46 potential compounds (Table 1) were investigated extensively. The majority of the detected metabolites are benzenedicarboxylic acid, cholest, guaiene, heptadecanoic acid, hexadecane, octadecane, octadecenoic acid, patchouli, pentanol, phenol, stigmast, tetradecanal, tetradecanoic acid, bicyclo, eicosanoic acid, pentacosadiynoic acid, pentadecanoic, oxirane, tetracosahexaene, heptadecane, hexadecanoic acid, and methoxycyclopropyl (Table 1). There were a few compounds discovered in this research. This discrepancy could be noticed since the technique's threshold for identification was higher than the quantity of compounds that were omitted. Additional investigation is necessary due to the potential complexity and selectivity of the unexplored compounds.

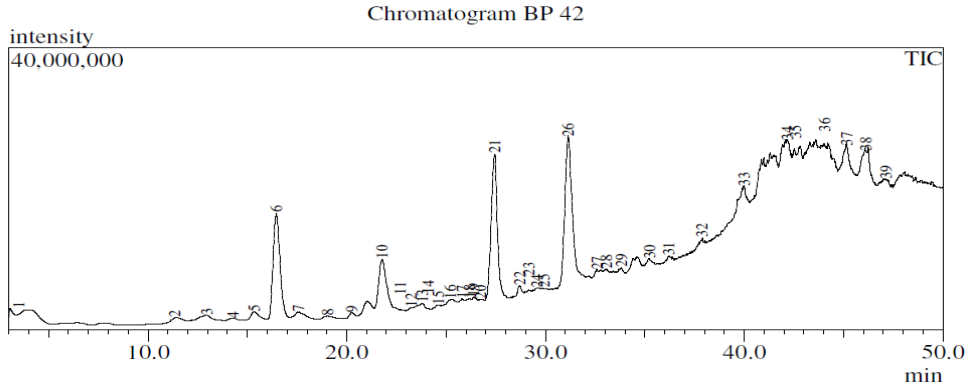

Fig. 2. Curves on the the spectrum generated from the BP 42 coffee clone's n-hexane segment

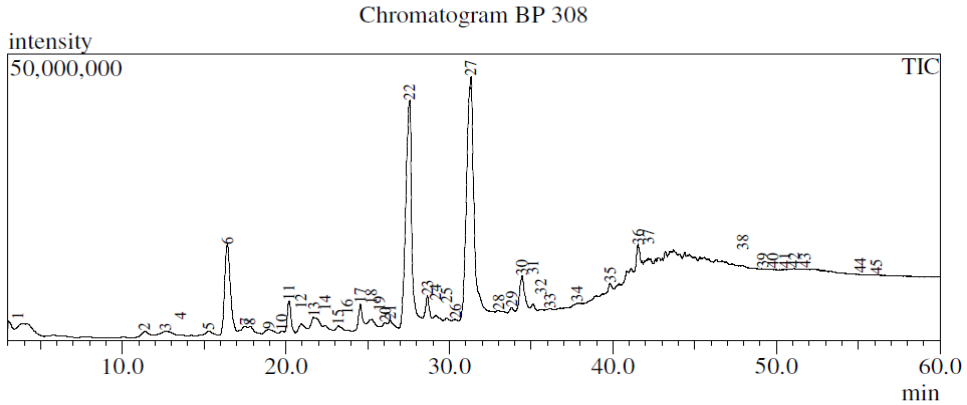

Fig. 3. Curves on the the spectrum generated from the BP 308 coffee clone's n-hexane segment

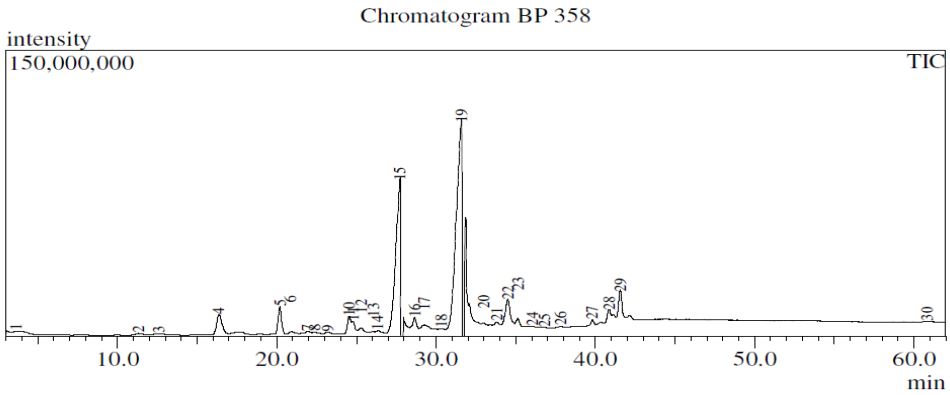

Fig. 4. Curves on the the spectrum generated from the BP 358 coffee clone's n-hexane segment

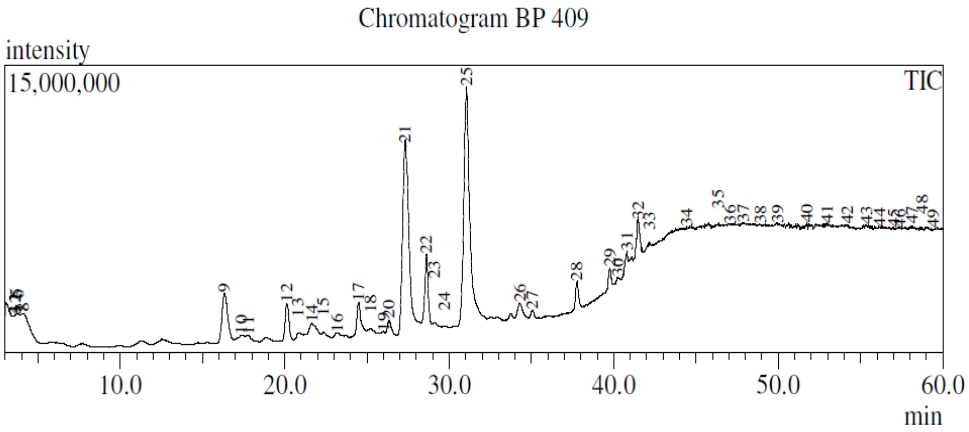

Fig. 5. Curves on the the spectrum generated from the BP 409 coffee clone's n-hexane segment

Table 1. Chemicals detected in the n-hexane section of coffee roots employing GC-MS

No.	Name of Chemical Substances	Formula	Molecular Weight	Class of Metabolites
1	Benzenedicarboxylic acid, bis(2-ethylhexy)	$C_{24}H_{38}O_4$	390,55	Benzoic acid esters
2	Benzenedicarboxylic acid, bis(2-methoxye)	$C_5H_8O_4S_2$	196,24	Fatty acids and conjugates
4	Benzenedicarboxylic acid, dinonyl ester	$C_8H_6O_7S$	246,19	Benzenesulfonic acids and derivatives
5	Cholest-5-ene, 3-bromo-, (3-beta)	$C_{27}H_{46}O_2$	402,65	Carbohydrates and carbohydrate conjugates
6	delta-Guaiene	$C_{15}H_{24}$	204,35	Sesquiterpenoids
7	Heptadecanoic acid (CAS) Margaric acid	$C_{17}H_{34}O_2$	270,45	Fatty acids and conjugates
8	Hexadecane (CAS) n-Hexadecane	$C_{19}H_{40}O_2$	300,52	Ethers

No.	Name of Chemical Substances	Formula	Molecular Weight	Class of Metabolites
10	Hexadecane, 2,6,10,14-tetramethyl (CAS) Ph	$C_{18}H_{38}O_2$	286,50	Ethers
11	Hexadecanoic acid (CAS) Palmitic acid	$C_{20}H_{40}O_2$	312,53	Diterpenoids
12	Octadecane (CAS) n-Octadecane	$C_{37}H_{65}NO_{14}$	747,92	Carbohydrates and carbohydrate conjugates
13	Octadecane, 1-chloro-(CAS) 1-Chlorooctadec	C_4H_9ClO	108,57	Alcohols and polyols
14	Octadecenoic acid	$C_{18}H_{34}O_2$	282,46	Fatty acids and conjugates
15	Patchouli alcohol	$C_{15}H_{26}O$	222,36	Alcohols and polyols
16	Pentanol, 2,3-dimethyl	$C_8H_{18}O$	130,22	Alcohols and polyols
17	Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl	$C_{20}H_{29}NO_3$	331,45	Phenylpropanes
18	Stigmast-5-en-3-ol, (3.beta.,24S)- (CAS) Clio	$C_{29}H_{50}O$	414,70	Stigmastanes and derivatives
20	Tetradecanal (CAS) Myristaldehyde	$C_{14}H_{28}O$	212,37	Fatty Aldehydes
21	Tetradecanoic acid (CAS) Myristic acid	$C_{14}H_{28}O_2$	228,37	Fatty acids and conjugates
22	Benzenedicarboxylic acid, 3-nitro (CAS)	$C_8H_6O_7S$	246,19	Benzenesulfonic acids and derivatives
23	Benzenedicarboxylic acid, bis(2-methylpr)	$C_{22}H_{25}Cl_2N_3O_2$	434,36	Benzoquinolines
24	Bicyclo(4.2.0)oct-1-ene, exo-7-(1-cyclohexen)	$C_{16}H_{17}N_9O_5S_2$	479,49	Beta lactams
25	Cholest-5-en-3-ol (3-beta)-(CAS) Lanol	$C_{45}H_{78}O_2$	651,11	Steroid esters
26	Dibenzo[a,h]cyclotetradecene, 2,3,11,12-tetrae	$C_{22}H_{14}$	278,34	not available
27	Eicosanoic acid (CAS) Arachidic acid	$C_{20}H_{40}O_2$	312,53	Fatty acids and conjugates
28	Hexadecane, 1-iodo	$C_{19}H_{40}O_2$	300,52	Ethers
29	Octadecanal	$C_{18}H_{36}O$	268,47	Fatty aldehydes
30	Octadecanoic acid (CAS) Stearic acid	$C_{18}H_{36}O_2$	284,47	Fatty acids and conjugates
32	Oxirane, 2,2-Dimethyl-3-(3,7,12,16,20)	$C_{11}H_{15}NO$	177,24	Phenylpropanes
33	Pentacosadiynoic acid	$C_{15}H_{24}$	204,35	Sesquterpenoids
35	Pentadecanoic acid (CAS) Pentadecylic acid	$C_{15}H_{30}O_2$	242,39	Fatty acids and conjugates
36	Tetracosahexaene, 2,6,10,15,1	$C_{30}H_{50}$	410,73	Triterpenoids
40	Dibutyl phthalate	$C_{16}H_{22}O_4$	278,34	Benzoic acids and derivates

No.	Name of Chemical Substances	Formula	Molecular Weight	Class of Metabolites
41	Eicosanoic acid	$C_{20}H_{40}O_2$	312,53	Fatty acids and conjugates
42	Heptadecane, 8-methyl	$C_{54}H_{96}O_6$	841,35	Triadylcglyserol
43	Methoxycyclopropyl-6,6-dimethyl-2,4-c	$C_{11}H_{16}O$	164,24	Monoterpennoids
44	Oxirane, tetradecyl	$C_4H_8O_3$	104,10	Alcohols and polyols
45	Hexadecanoic acid, 1,2,3-propanetriyl ester	$C_{17}H_{32}O_2$	268,43	Fatti acid esters
46	Octadecenoic acid (Z)-(CAS) Oleic acid	$C_{18}H_{34}O_2$	282,46	Fatty acids and conjugates

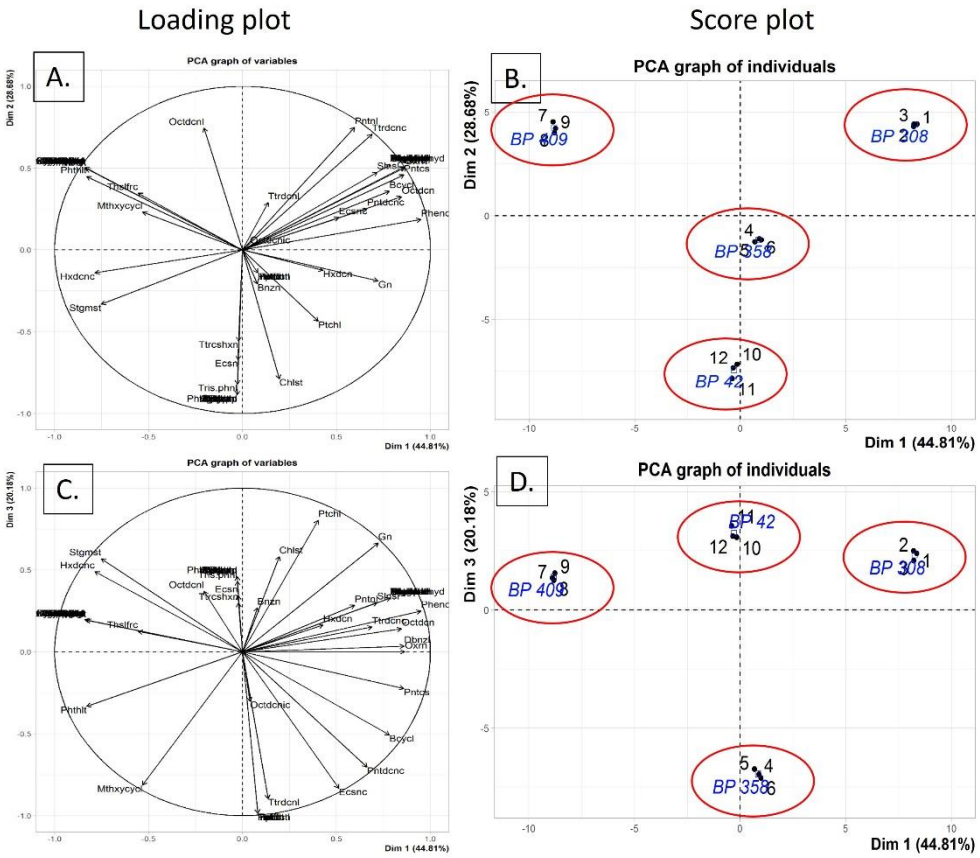

Fig. 6. Principal Component Analysis on Metabolic Degree Associations. Loading plots (A and C) and Score plots (B and D) are shown for combinations of PC1, PC2, and PC3. PCA was performed on combined non-redundant data involving three replicates of the levels of metabolite compounds in revealing the defense mechanism between resistant and susceptible coffee varieties by using Rstudio software. In the loading plots A and C, the eigenvectors represented by black arrows show how (the direction) and how much (the length) each metabolite contributes to the individual correlations represented by PC1, PC2, and PC3. In the score plots B and D, the black-coded numbers represent the corresponding varieties of coffee (BP 308, BP 358, BP 409, and BP 42),

and each dot of the same cluster of coffee represents replicate samples. The ovals highlight the basic clustering/separation of similar/dissimilar samples.

Untargeted GC-MS results revealed secondary metabolite profiles of resistant and vulnerable coffee species to nematode. Based on the arrangements of the biological replications in the research measurements it was analytically demonstrated that the resistant and susceptible categories had unique chemical compositions. Divergences in the number of chemicals pooled among the tolerant and vulnerable populations were discovered utilizing an analysis of multivariate statistics indicating as marker compounds. Moreover, we conducted principal component analysis (PCA) on the relationships between the quantities of chemicals to acquire an overall understanding of these biochemical compound-related defense mechanisms. The data matrix used for classification had the dimension of 12 monitoring points and 46 measured variables. The first three principal components (PCs) represented 93.67% (PC1: 44.81%, PC2: 28.68%, PC3: 20.18%) of the overall disparity between the samples (Fig. 6). The mixtures of PC1-PC2 and PC1-PC3 allowed coffee varieties illustrating various primary chemical reactions to be easily recognized from other varieties (Fig. 6).

PCA evaluation using the loading plot discovered potential metabolite chemicals that serve as marker chemicals that can differentiate among species both sensitive yet immune to nematodes. Each coffee variety has different metabolic compound characteristics. In combination with PC1-PC2 (Fig. 6A), BP 409 was characterized by Octadecanal (Octdcnl) and Tetradecanal (Ttrdcnl). BP 308 has been recognized as Phenol and Guanine. BP 358 and BP 42 have been described as Cholest (Chlst) and Patchouli (Ptchl). The characteristics of these metabolic compounds function as specific marker compounds in coffee varieties that are resistant and susceptible to nematodes. The movement of the arrow on the attribute plot, which connects to the graph of the resistant and susceptible varieties, was used to determine these chemicals. Those substances were in the same region as the variety, according to the loading and score plots.

Biplots and score plots from PCA evaluation can be used to determine the corresponding varieties of coffee (BP 308, BP 358, BP 409, and BP 42) by clustering/separating similar/dissimilar samples between nematode-resistant and susceptible varieties. BP 308, BP 358, BP 409, and BP 42 were metabolically distinct from one another based on combination with PC1-PC2 (Fig. 6B). Each of the varieties (BP 308, BP 358, BP 409, and BP 42) clustered separately based on both PC1 and PC2, suggesting that these varieties are metabolically different from other varieties. The samples from resistant (BP 308) and susceptible (BP 358, BP 409, and BP 42) groups were separated.

Combinations with PC1-PC2 have higher values (44.81% and 28.68%) than combinations with PC1-PC3 (44.81% and 20.18%). These findings suggest that PC2 most accurately conveys the chemical alterations that are employed throughout the defensive response. However, all the coffee varieties appeared metabolically different from other varieties based on the loading plot of PC3 (Fig. 6C) and all the coffee varieties separation could also be observed based on the score plot of PC3 (Fig. 6D). Nevertheless, the first two PCs indicate very clearly the metabolic reprogramming occurring during the defense mechanism.

4 Conclusions

The mixtures of PC1, PC2, and PC3 allowed coffee varieties that exhibited several primary biochemical reactions to be quickly recognized from other varieties. Each of the varieties (BP 308, BP 358, BP 409, and BP 42) clustered separately based on both PC1 and 2 and PC1 and 3, suggesting that these varieties are metabolically different from each other. BP 409 was

differentiated by Octadecanal and Tetradecanal, BP 308 by Phenol and Guaiene and BP 358 and BP 42 by Cholest and Patchouli.

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