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Unraveling metabolite profile variations among resistance and susceptible shallot genotypes related to anthracnose (*Colletotrichum gloeosporioides*)

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Abstract. *Amrullah RA, Maharijaya A, Purwito A, Wiyono S. 2023. Unraveling metabolite profile variations among resistance and susceptible shallot genotypes related to anthracnose (*Colletotrichum gloeosporioides*). Biodiversitas 24: 5113-5122.*This study used a metabolomics approach to reveal the shallot metabolite profile under anthracnose disease conditions through GC-MS analysis. The aim of this study was to obtain candidate metabolite markers that differentiate shallot genotypes resistant to anthracnose. The results of non-targeted metabolite analysis with GC-MS revealed a shallot metabolite profiles of 54 compounds. The principal component analysis (PCA) model was conducted for reliable and accurate discrimination between uninoculated and inoculated genotypes. Four metabolites were revealed as putative biomarkers of *Colletotrichum gloeosporioides* Penz. infection, namely linoleic ethyl ester, squalene, octadecanoic acid, and nonacosane. At least some putative biomarkers were applicable for early resistance genotype detection in shallot. These metabolites may help characterize pathogen infection and plant defense responses. This study confirmed metabolomics as a tool to develop a strategy to clarify the mechanism of plant-pathogen interaction. Furthermore, the data presented may be helpful to developing a new method for detecting shallot, that may be resistant to anthracnose caused by *C. gloeosporioides*.

Keywords: Biomarker, *C. gloeosporioides*, detection, GC-MS, principal component analysis, shallot

INTRODUCTION

Shallot (*Allium cepa* L.) is an agricultural commodity that is widely cultivated in various countries in the world. Shallots are a seasoning product with high nutritional value because of their antioxidant content, which can reduce the risk of cancer, DNA damage, and heart attack (Sidhu et al. 2019). Globally, the demand for shallot was sixth place after pepper, and the market is dominated by China (23.72%) and Japan (15.24%) (Chappell and Dunford 2021). However, the cultivation of shallots in recent years has experienced severe disturbances caused by disease. Anthracnose disease caused by *Colletotrichum gloeosporioides*, which has never been reported on shallots, especially on leaves, has begun to be reported in the last few decades. This disease is one of the major diseases in shallots in several countries, such as the Philippines (Alberto et al. 2001), Indonesia (Hidayat and Sulastrini 2016), and Georgia (Nischwitz et al. 2008).

Anthracnose was detected in shallots in 1969 in Zaria, Northern Nigeria (Ebenebe 1980). Appropriate environmental conditions, such as temperature and humidity, can significantly threaten the possibility of anthracnose outbreaks in shallots (Wiyono 2007). The pathogenicity of *C. gloeosporioides* can vary in growth phases and different plant parts, as in the vegetative phase, it has symptoms of spotting, necrosis, chlorosis, and abnormal growth. Shallot bulbs produced when infected with *C. gloeosporioides* are smaller in size with low number and weight (Hekmawati et al. 2018). *C. gloeosporioides* infects plants through tissues by penetrating the cuticle and destroying the cell wall to enter inter and intracellular tissues. It secretes hydrolytic enzymes during the infection process. Hence, cell organelles are damaged, starting from the chloroplasts and ending with damage to the entire cell (Armesto et al. 2019).

Plants have a strategy for carrying out defense against disease. Plant strategies for tolerating disease can differ between genotypes. Plant responses to disease are complex and require interactions among many metabolites. The composition of metabolites in plants varies greatly and can change because different physiological and environmental conditions influence it. In addition, the composition of these metabolites can describe the genetic background of a plant. Differences in response and the resulting metabolites can be used to select disease-resistant plants (Chen et al. 2019). Several studies have reported differences in responses between plants, such as linoleic acid being an essential compound in the defense mechanism of soybeans against *Rhizoctonia solani* (Aliferis et al. 2014). Strawberries infected with anthracnose by *Colletotrichum theobromicola* also have marker compounds when infected, such as shikimic acid and malic acid compounds (Dai et al. 2019). An increase in pyruvic acid and its derivatives indicates that shallots are infected with *Stemphylium vesicarium* (Medina-Melchor et al. 2022). However, studies regarding

the metabolite profile indicating that shallots are resistant to anthracnose have yet to be found.

Metabolomics approach provide the opportunity to be able to reveal the biochemical processes that occur in a plant with a variety of different genetic backgrounds. Gas chromatography-mass spectrometry (GC-MS) or Liquid chromatography-mass spectrometry (LC-MS) can be used to describe the profile of untargeted metabolites (Perez de Souza et al. 2019). Profiling of secondary metabolites is a bioanalytic method that can study changes in metabolites in response to pathogen infection. The profile of plant metabolites infected with a disease could be analyzed using the gas chromatography-mass spectrometry method (Medina-Melchor et al. 2022). The purpose of detecting non-targeted metabolites to obtain as much data as possible from the metabolites detected in the tested samples (Hanifah et al. 2018). Using a metabolomics approach through GC-MS analysis, the aim of this study was to obtain candidate metabolite markers that differentiate shallot genotypes resistant to anthracnose.

MATERIALS AND METHODS

Plant material and fungal strain

Colletotrichum gloeosporioides used in this study was collected from the Plant Clinic, IPB University, isolated from shallot. The four shallot genotypes used in the metabolomics profiling experiment, namely Rubaru, Batu Ijo (resistant), Biru Lancor, and Bima Brebes (susceptible), were obtained from breeder farmers in Brebes, Central Java. *C. gloeosporioides* was cultured on a PDA medium, incubated at 25°C until acervulus appeared and covered the entire medium surface. The spore suspension was prepared from fully grown fungus having acervulus. Afterward, 100 mL of sterilized water was added, and the mixture was thoroughly agitated. The resulting solution was then filtered using a millipore syringe filter and subsequently diluted to a concentration of 1 to 1.5 million conidia per milliliter (1-1.5x10^6 conidia.mL^{-1}). The composition of the growing media was a mixture of sand and soil with fertilizer in a 1:1 (v/v) ratio, and it was autoclaved at 121°C for 45 minutes. The bulb was surface sterilized using 70% ethanol for 5 min, rinsed with sterilized water, and then treated with hot water at 50°C for 15 min.

Disease severity experimental design

For the assessment of disease severity, Rubaru and Batu Ijo (resistant) and Biru Lancor and Bima Brebes (susceptible) varieties were separately grown in artificial inoculation conditions along with the metabolomic experiment. The experiment was conducted in a randomized complete block design with three replicates in each unit. Artificial inoculation was carried out by spraying the plants after growing them for 31 days using spore suspension. The disease was scored weekly to determine the disease severity (%), as described by Allen (1983). The formula used to calculate disease severity was:

$$DS = \frac{\sum(n_i \times v_i)}{Z \times N} x\ 100\%$$

Where: DS: Disease severity, n_i: Number of infected plants, v_i: Infected plant score category, Z: The highest category score, and N: Number of observed plants. An ordinal scale was used to score the individual plant reaction to fungus: 0: no symptoms; 1: oval-shaped white spots on leaves, 1-5 spots present; 2: large oval-shaped white spots, 1-2 large spots present; 3: sunken necrotic white spots with the appearance of acervuli; 4: appearance of concentric orange acervuli spots, leaves start to turn yellow; 5: dark concentric acervuli spots causing death in leaf tissue.

Metabolomic profiling experimental design

The plant material was grown for a single time point (8 weeks). The experiment consisted of eight treatments: (i) Rubaru inoculated with *C. gloeosporioides*; (ii) Rubaru uninoculated; (iii) Batu Ijo inoculated with *C. gloeosporioides*; (iv) Batu Ijo uninoculated; (v) Biru Lancor inoculated with *C. gloeosporioides*; (vi) Biru Lanco' uninoculated; (vii) 'Bima Brebe' inoculated with *C. gloeosporioides*; (viii) 'Bima Brebes uninoculated. One replicate consisted of two plants per polybag. Three replicates per treatment were grown in a randomized complete block design (RCBD). Three replicates were used for untargeted metabolic profiling using an untargeted GC-MS protocol.

Plant growth and treatment conditions

Plants for the metabolomic experiment were grown in a controlled, transparent fabric hood to maintain humidity. Inoculation was carried out 31 days after planting (3-5 leaves). The plants were inoculated in the evening by spraying 48 mL suspension, and the control plants (uninoculated) were sprayed with distilled water. The hood was sprayed with water, and a plastic film sealed each pot to maintain moisture. The plastic film was removed after two days. Leaf samples were collected 48 hours after inoculation with three replicates per treatment. For each replicate, leaves were cut using sterilized scissors. The leaves were rinsed with distilled water to remove residual, dried and then stored at -80°C.

Metabolome sample extraction

The extraction of metabolites was carried out using the maceration method. Metabolites were extracted using methanol pro analysis at 25°C for 5 d (1 g sample:10 mL solvent). The extracts were then evaporated at 45°C using a turbo vap evaporator for one hour.

Untargeted metabolomic analysis

The analysis was performed on a 0.11 µm capillary column (HP Ultra 2). In the detection device, the injection temperature was set at 250°C, the ion source temperature was 230°C, interface temperature was 280°C, and the quadrupole temperature was 140°C. Helium was used as the carrier gas at a flow column of 1.2 mL/min with an injection volume of 5 µL and a split ratio of 8:1. The initial analysis temperature was 80°C which was then increased to 150°C for 1 minute at a speed of 3°C/minute. Temperature was increased to 280°C for 26 minutes at a 20°C/minute speed. The mass spectrum was detected in the 20-500 m/z mass-to-charge range. Data from the scanning tool identified

the compound's name based on the Wiley W8N08.L database.

Data processing and statistical analysis

The metabolites obtained were identified as the group compound based on online databases, i.e., ChemSpider (http://www.chemspider.com/), PubChem (https://pubchem.ncbi.nlm.nih.gov/), Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://genome.jp/kegg/pathway.html). Chromatograms were aligned using OriginPro2023. The data obtained was a percentage area (%) and then used for further analysis. The data that obtained was then processed by selecting secondary metabolites with quality >80%. The data was transformed and scaled using the mean centering method. The metabolite data were analyzed using univariate and multivariate analysis using MetaboAnalyst 5.0 to identify metabolite markers for anthracnose resistance response in shallot. Heatmap clustering analysis (HCA), principal component analysis (PCA), biplot analysis, and analysis of variance (ANOVA) were performed to determine the essential metabolite markers that can differentiate between the two groups of plant resistance. The Venn diagram was produced using InteractiveVenn (http://www.interactivenn.net/).

RESULTS AND DISCUSSION

Shallot genotypes resistance to anthracnose

Symptoms of anthracnose disease (Figure 1) and changes in disease severity at weeks 1, 2, and 3 (Table 1) confirmed the infection of *C. gloeosporioides*. Genotypes were tested for significant ($p \leq 0.05$) differences in performing anthracnose susceptibility. Biru Lancor showed the highest (24.89%) disease severity at the first week and at the end of observation (62.44%). The first symptoms appeared on the second day after inoculation on the Biru Lancor. The resistant genotype, Rubaru, showed the lowest (8.85%) disease severity compared to the other genotypes at the first week and at the end of observation (27.61%). Two groups of resistant and susceptible genotypes were observed in the third week. Rubaru and Batu Ijo showed the low disease severity values indicating resistant genotypes, while Bima Brebes and Biru Lancor showed high disease severity values indicating susceptible genotypes.

Metabolite profile of four shallot genotypes under anthracnose inoculation

The results of GC-MS analysis were expressed in the peak form, representing different compounds. A total peaks of the detected compounds reached 492 peaks from all treatments and were reduced based on the selection of the percentage area and the quality of the compounds to 54 compound peaks. Identification results based on the database showed a diversity of compounds between treatments. Several compounds, such as Neophytadiene, Vitamin E, and Phytol, were found in almost all treatments (Figure 2). The detected metabolites were compounds derived from the group of hydrocarbons, fatty acids, and other metabolites.

The findings of untargeted GC-MS analysis using methanol solvent extraction of shallot leaf extracts from anthracnose-resistant and susceptible genotypes showed a total of 54 compounds, of which 29 were identical between the uninoculated and inoculated conditions (Figure 3). The highest number of known compounds (38 metabolites) were found in Rubaru, followed by Bima Brebes (36 metabolites), Batu Ijo (32 metabolites), and Biru Lancor (21 metabolites). The resistant genotypes exhibited a higher response in producing metabolite compounds (Batu Ijo 18 metabolites and Rubaru 22 metabolites) when under inoculated conditions compared to the susceptible genotypes (Bima Brebes 12 metabolites and Biru Lancor 3 metabolites).

Heatmap cluster analysis (HCA) showed that the samples formed two main groups based on the secondary metabolite profile (Figure 4). The two groups were mostly filled with similar resistance statuses. The susceptible group was in the first cluster, and the resistant group was in the second cluster. Differences in grouping were caused by variations in the response of each variety to anthracnose disease, as indicated by differences in the composition of secondary metabolite compounds produced.

Table 1. Disease severity across the genotypes at 1, 2, and 3 weeks after inoculation

Genotypes	Disease severity (%)		
	1st week	2nd week	3rd week
Rubaru	8.85 b	16.26 b	27.61 b
Batu Ijo	15.71 ab	22.14 b	29.52 b
Bima Brebes	19.84 ab	43.65 a	59.84 a
Biru Lancor	24.89 a	33.74 ab	62.44 a

Note: Different letters indicate significant differences ($p>0.05$) between genotypes

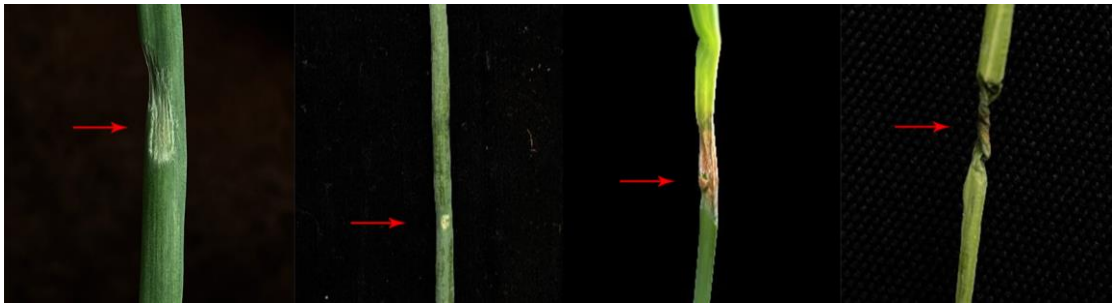

Figure 1. Symptoms of anthracnose disease on shallot leeks were observed 3 weeks after inoculation. A. Batu Ijo, B. Rubaru, C. Bima Brebes, D. Biru Lancor. The red arrow indicates the symptoms

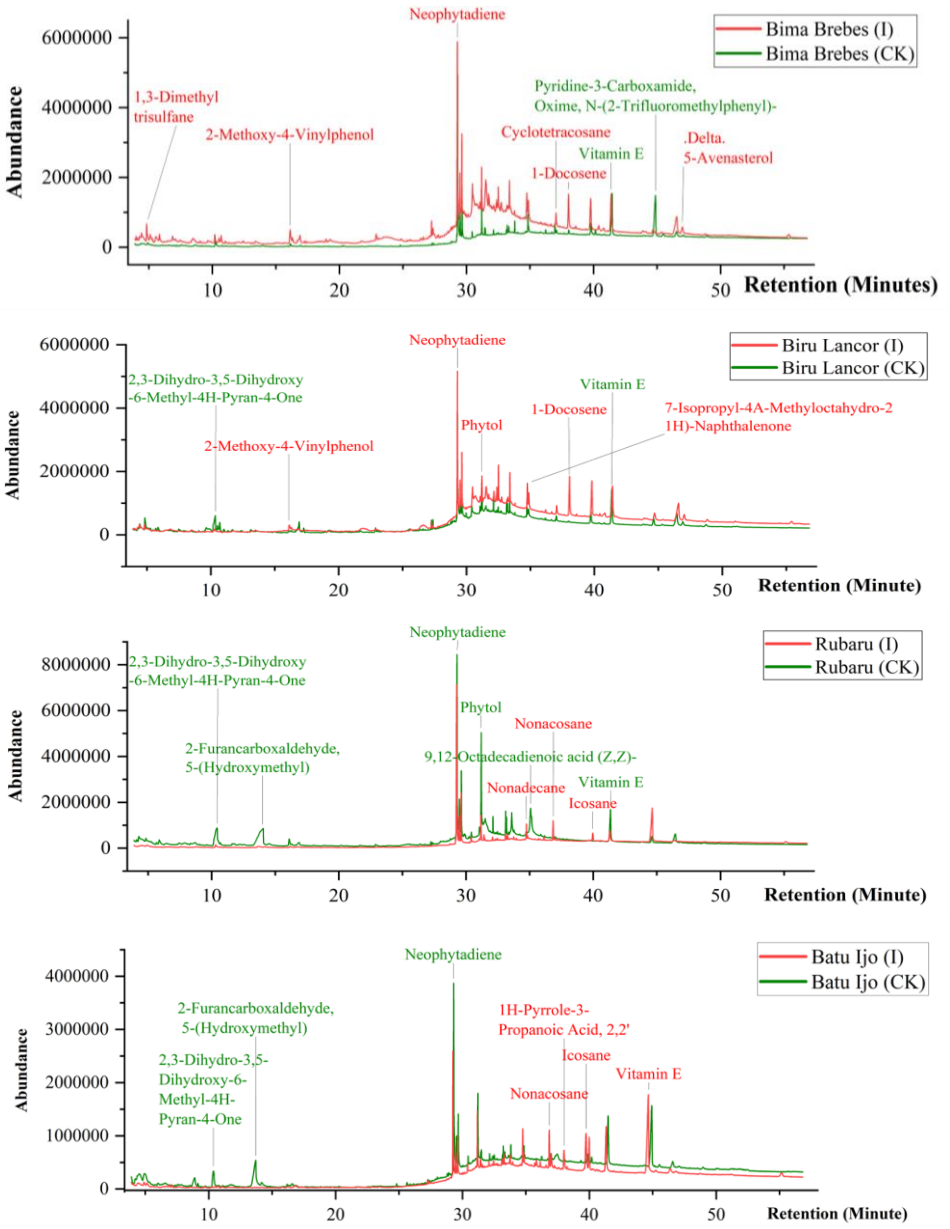

Figure 2. Representative gas chromatogram-mass spectrometry total ion chromatograms of metabolome extracted from four shallot genotypes inoculated (I/red line) or uninoculated (CK/green line)

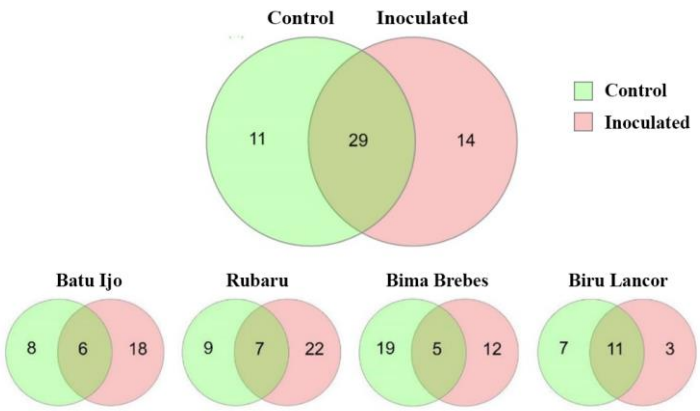

Figure 3. Comparison of shallot metabolites number under uninoculated and inoculated anthracnose conditions

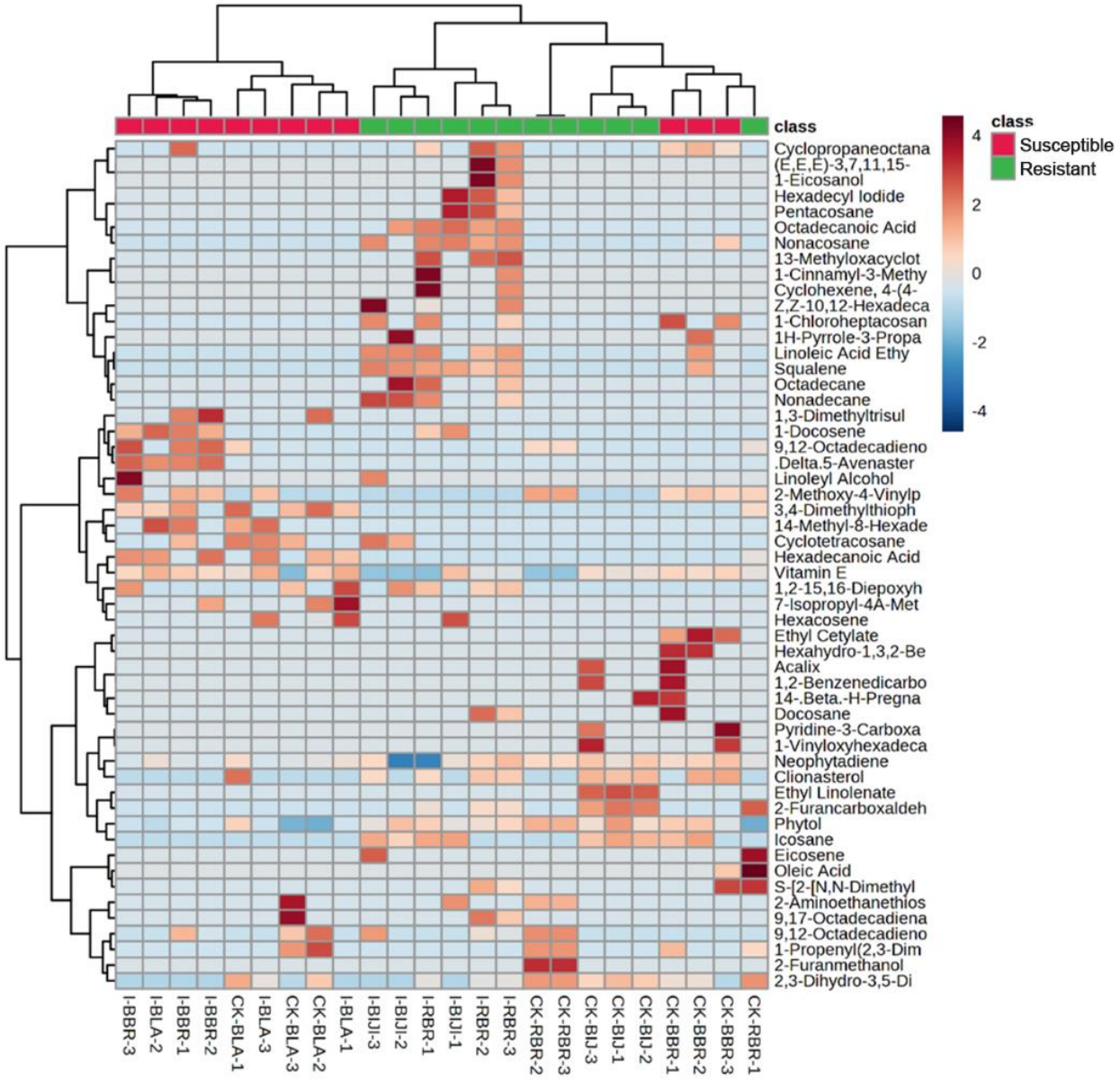

Figure 4. Profile of shallot metabolites under uninoculated and inoculated anthracnose conditions. CK: Uninoculated, I: Inoculated, BBR: Bima Brebes, BLA: Biru Lancor, BIJ: Batu Ijo, RBR: Rubaru

Identification of putative metabolite marker for anthracnose resistance response in shallot

The metabolomic data from each treatment sample (n=24) were used as subjects of the PCA model (Figure 5). Based on the PCA analysis of the metabolite compound profile, the samples from inoculated and control groups were separated, as well as resistant and susceptible groups. PC1 and PC2 values explained 37.5% and 15.5% of the variation, respectively, with a total explained variation of 53.0% for the shallot metabolite compounds. Marker compounds were specific compounds that characterized or differentiated the anthracnose-resistant shallot genotypes. Marker metabolite compounds in this study were identified using two multivariate statistical approaches (PCA analysis). PCA analysis using score plots and biplots can help identify potential metabolite compounds that serve as marker compounds. The inoculated resistant genotype samples (Batu Ijo and Rubaru) were clearly separated from the susceptible genotype samples (Bima Brebes and Biru Lancor).

PCA analysis using the loading plot identified compounds that can distinguish between anthracnose-resistant and susceptible varieties. Rubaru and Batu Ijo varieties had four compounds that distinguished them from susceptible varieties. These compounds were identified based on the direction of the vector on the feature plot that leads to the plot of the inoculated resistant varieties (Figure 6). Based on the score plot and loading plot, these compounds were in the same quadrant as the resistant varieties under inoculated conditions. The metabolites

compound responsible for maximum separation in PC1 and PC2 axes, respectively, to their PCA loading, are given in Tables 2 and 3. The metabolite compounds with the highest positive value on the PC1 axis (Table 2) represent the unicolated and inoculated resistance genotypes (Rubaru and Batu Ijo). The metabolite compound with the largest negative value on the PC1 axis represents the susceptible uninoculated and inoculated genotypes (Biru Lancor and Bima Brebes). Metabolites on the positive PC2 axis (Table 3) represented inoculated treatment for all genotypes. However, metabolites on the most negative side of PC2 were more responsible for separating uninoculated treatment for all genotypes. Fatty acid compounds have many roles in the performance of compounds that appear in inoculated-resistant varieties, especially in the positive PC1 axis.

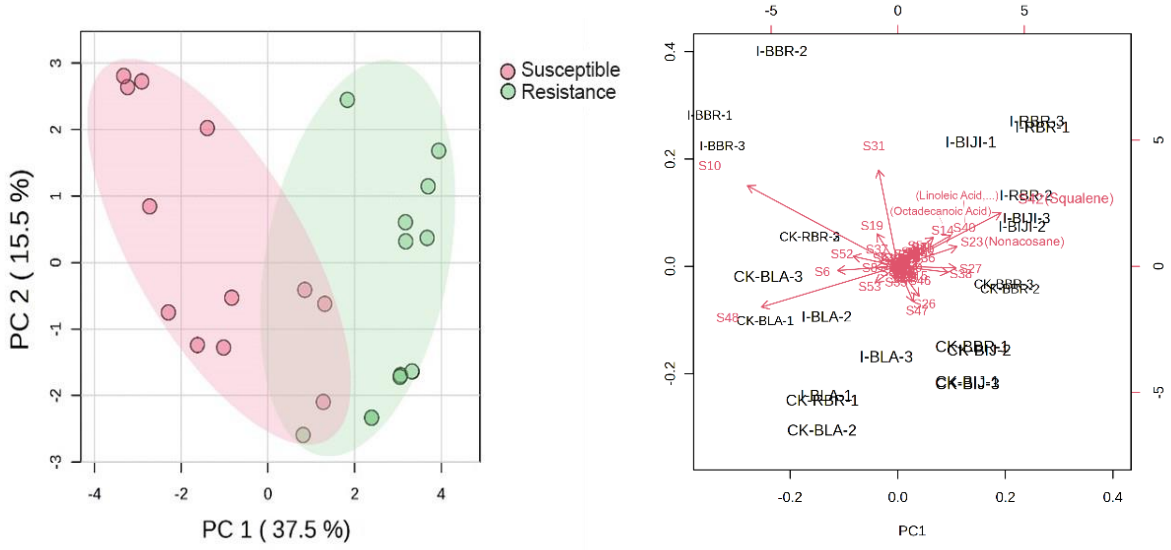

Figure 5. PCA graph of shallot resistance to anthracnose in uninoculated and inoculated conditions. Left: score plot; Right: loading plot. CK: Uninoculated, I: Inoculated, BBR: Bima Brebes, BLA: Blue Lancor, BIJ: Batu Ijo, RBR: Rubaru

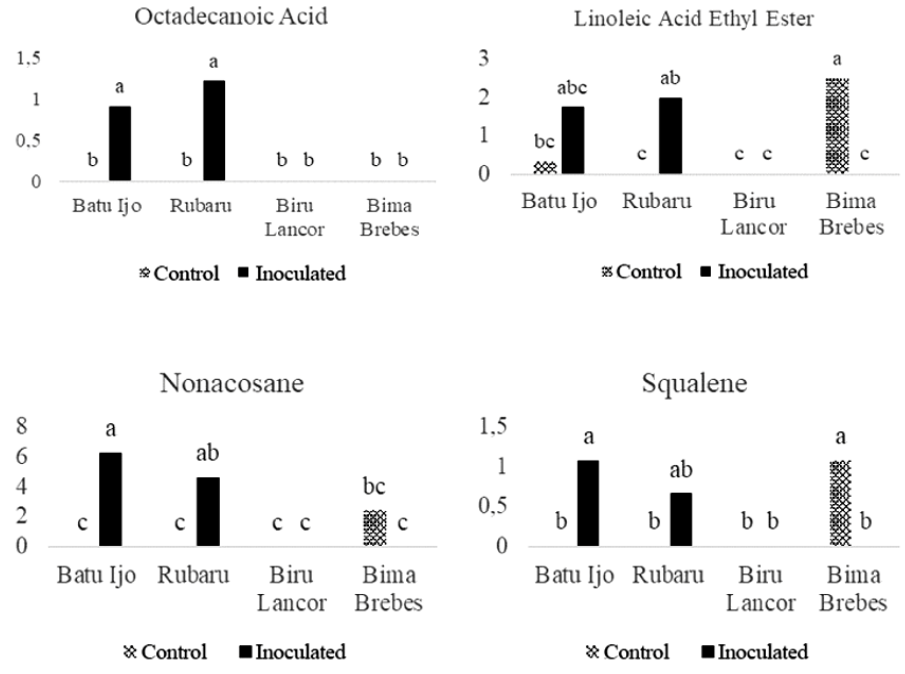

Figure 6. Comparison of compounds' percentage area (x-axis) between varieties (y-axis) under uninoculated and inoculated conditions. Based on the Tukey test, bars with the same letter are not significantly different at the 5% level

Table 2. Metabolites with the highest positive loadings and highest negative loadings along principal component 1 (PC1) from the principal component analysis of secondary metabolites percentage area (%)

Metabolite on PC1 axis[a]	RT	Groups	Metabolites on PC1 axis[b]	RT	Groups
Linoleic acid ethyl ester	31.406	Fatty acid	3,4-Dimethylthiophene	48.385	Sulfur-containing
Squalene	36.122	Terpene	9,12-Octadecadienoic Acid (Z,Z)-	31.054	Fatty acid
Octadecanoic acid	30.406	Fatty acid	1-Propenyl(2,3-Dimethylthien-5-Yl) Disulfide	27.365	Sulfur-containing
Nonacosane	36.846	Hydrocarbon	2-Methoxy-4-Vinylphenol	16.126	Phenol

Note: a: most positive; b: most negative; RT: Retention time (minutes)

Table 3. Metabolites with the highest positive loadings and highest negative loadings along Principal Component 2 (PC2) from the principal component analysis of secondary metabolites percentage area (%)

Metabolite on PC2 axis[a]	RT	Groups	Metabolites on PC2 axis[b]	RT	Groups
1-Docosene	34.833	Hydrocarbon	2,3-Dihydro-3,5-Dihydroxy-6-Methyl-4H-Pyran-4-One	10.306	Hydroxyl
Squalene	36.122	Terpene	1-Propenyl(2,3-Dimethylthien-5-Yl)Disulfide	10.320	Sulfur-containing
Hexadecanoic acid	30.475	Fatty acid	Clionasterol	46.458	Phytosterol
3,4-Dimethylthiophene	48.385	Sulfur-containing	2-Furancarboxaldehyde, 5-(Hydroxymethyl)	13.740	Aldehydes

Note: a: most positive; b: most negative; RT: Retention time (minutes)

Based on the semi-quantitative PCA analysis, the inoculated resistant genotypes of the four compounds with the highest PC1 axis values were further identified for different compounds between treatments. The four compounds in the inoculated resistant genotypes showed increased value. Linoleic acid ethyl ester, nonacosane, and Squalene (Figure 6) contributed most to PC1, significantly increasing in inoculated Batu Ijo and Rubaru genotypes. However, the genotype Bima Brebes showed increased compounds in uninoculated conditions. However, octadecanoic acid was found to be increased only in the treated resistant genotypes.

Discussion

Associated metabolite marker to resistance

This is the first study to investigate the metabolite profile variation of resistance and susceptibility shallot genotypes inoculated with anthracnose caused by *Colletotrichum gloeosporioides*. The non-targeted metabolomics approach used in the present study comprehensively revealed the profile of metabolites in inoculated plants 48 hours after inoculation, revealing the profile of plant's active resistance. The characteristic symptoms of anthracnose were shown differently in the four shallot varieties tested in the second week of observation. The lesions that formed on the Biru Lancor variety were the most severe, with a twisted brown color indicating that the plant tissue had died. Further higher severity cause wider lesion and tissue to become damaged, leading to death of leaves (Dutta et al. 2022). Moreover, 48 hours after inoculation, hyphae enter the tissues through open stomata of the leaves and form a mass of conidia (Panday 2012). So, in this phase, evaluating the response of plant resistance through a metabolomics approach from varieties resistant and susceptible to anthracnose can detect forms of active plant resistance. Metabolomics is a valuable tool in evaluating the adaptation forms of plants to infection with disease pathogens (Lowe-Power et al. 2018). Metabolomics analysis of shallots related to disease resistance has been carried out on several diseases such as *Fusarium* (Abdelrahman et al. 2020) and flowering activity (Marlin et al. 2019).

Untargeted GC-MS results revealed secondary metabolite profiles of shallot genotypes resistant and susceptible to anthracnose. The difference in metabolite profiles between the inoculated and uninoculated treatments was statistically proven based on the grouping of the biological replicates in the experimental samples. Multivariate statistical analysis revealed differences in the number of compounds clustered between resistant and susceptible samples. PCA revealed good clustering between resistant and susceptible genotype samples and their association with metabolites. This difference also reveals an increase in metabolite activity when anthracnose pathogens are inoculated. Stress conditions result in an increase in the number of metabolites formed. Varieties with resistance status (Batu Ijo and Rubaru) showed increased metabolites when inoculated with anthracnose. In contrast, susceptible varieties (Biru Lancor and Bima Brebes) exhibited a decrease in the number of responses of secondary metabolites. There was an increase in the number of secondary metabolites in the elephant grass family, which was artificially inoculated with *Fusarium culmorum* (Piasecka et al. 2022). Rubaru is the most resistant shallot genotype based on disease severity analysis. Based on PCA analysis, compounds were statistically associated with resistance genotypes (Rubaru and Batu Ijo). Furthermore, there was also a significant difference based on the analysis of disease severity (%) where Rubaru and Batu Ijo were significantly different

from 'Biru Lancor' and 'Bima Brebes.' The different groups formed and associated with the expressed metabolites indicate a link between anthracnose inoculation and the resistance of the Rubaru and Batu Ijo genotypes. In the present study four marker compounds, based on their association with the inoculated resistant genotype treatment characterized by a significant contribution to the value of the PC1 axis, namely octadecanoic acid, linoleic acid ethyl ester, nonacosane, and squalene. These metabolites strongly contributed to the separation between resistant and susceptible genotypes under inoculated conditions. This finding indicates that these metabolites play an essential role in shallot resistance to *C. gloeosporioides.*

Metabolite effect on anthracnose resistance/susceptibility performance

This study identified compounds related to plant resistance based on position in the score plot and loading plot quadrants. These compounds were octadecanoic acid, linoleic acid ethyl ester, nonacosane, and squalene. For comparison, the susceptible variety appeared as a marker compound under control conditions but was not detected under inoculated conditions. This indicates that compound was inducible by *C. gloeosporioides* inoculated treatment. The compound that was generally found in resistant varieties under inoculated conditions was octadecanoic acid. However, PCA analysis revealed that these four compounds were related to disease resistance, as shown in the vector loading plot, forming quadrant of the inoculated resistant variety samples. Octadecanoic acid is reported to be a precursor of jasmonic acid, an essential signal-inducing molecule in response to biotic stress by pathogens (Coleman and Hudson 2016). Linoleic acid ethyl ester is reported to have a role as an antifungal compound (Maia et al. 2022) and antibacterial (Naser et al. 2019). Nonacosae compounds are reported to increase up to six times in arabidopsis plants infected with the fungus *Botrytis* sp. (Reina-Pinto and Yephremov 2009). Inducible means that the marker compounds appear under certain conditions. In this study, these compounds were found in varieties that resistant to anthracnose but have been exposed to conditions where they were inoculated with the fungus, resulting in these compounds being inducibly responded.

Octadecanoic acid (IUPAC term), or the stearic acid compound, is a fatty acid group reported as a compound with anti-inflammatory properties. Octadecanoic acid is reported to have upregulated activity in sorghum plants' roots, leaves, and stems when infected with *Fusarium pseudograminearum* (Carlson et al. 2019). Qi et al. (2015) reported that octadecanoic acid was increased after inoculation treatment of leaf rust disease on barley plants. It has also been reported to be a precursor of several other secondary metabolites, such as linoleic acid, a precursor in hormone signaling. In particular, compared with other marker metabolites in the treatment of resistant varieties in this study, which were inoculated with the anthracnose pathogen *C. gloeosporioides*, it showed an increase/appearance of activity compared to the control treatment.

Linoleic acid ethyl ester, a marker compound for shallot resistance to anthracnose, is an unsaturated fatty acid derivative of linoleic acid. Linoleic acid ethyl ester is a dissolved form of the compound linoleic acid. In potatoes, linoleic acid ethyl ester was reported as a secondary metabolite compound that forms systemic resistance to *Phytophthora infestans* through octadecanoic activation to produce jasmonic acid, initiates the production of resistance proteins (Cohen et al. 1991). Linoleic acid, is a substrate of lipoxygenase enzyme (Singh et al. 2022). Lipoxygenase is a product of the LOX gene, which is reported to play a role in the mechanism of potato resistance to *Phytophthora infestans* in the early stages of infection (Viswanath et al. 2020).

Nonacosane compounds are reported to play a role in modifying plant tissues, especially in forming a wax layer in the cuticle of the epidermis when an infection occurs by a fungal pathogen (Lykholat et al. 2021). There is a correlation between resistance to fungal pathogens and the production of nonacosane, whose role is to inhibit the growth of the fungus appressorium. About 44% of nonacosane compounds comprise the wax layer after inoculating *Alternaria alternata* on Asian Pear plant tissue (Yin et al. 2011). In this study, it was discovered that when *C. gloeosporioides* was inoculated to resistant varieties, a set of nonacosane compounds was triggered. Interestingly, these compounds were also observed in the Bima Brebes as susceptible variety under control conditions. This finding strongly suggests that the Bima Brebes variety has lost its ability to prevent the germination of *C. gloeosporioides* appressorium, which is typically a defense mechanism against the fungus.

Squalene is a compound that comes from the triterpenoid group and acts as a hormone precursor and secondary metabolite compound (Ghimire et al. 2016). Squalene is also reported to be found in many plants, such as olives (564 mg/100 g), soybeans (9.9 mg/100 g), wheat (14.1 mg/100 g), peanuts (27.4 mg/100 g), and spinach (5942 mg/100 g) (Lozano-Grande et al. 2018). Characterization of volatile compounds carried out by Lekshmi et al. (2014) using the GC/MS technique on *Allium cepa* tuber extracts also found squalene compounds. Squalene compounds were reported in the treatment of *Allium sativum* infected with *Sclerotium cepivorum* by Pontin et al. (2015) and experienced an increase compared to control from 11.0% to 33.8%. Squalene was reported to be a compound involved in the WRKY1 gene expression pathway, a resistance gene, in *Withania somnifera* plants where there was an increase in the amount of squalene by 213% compared to the control treatment (Singh et al. 2017). This study found that the identified secondary metabolites (Octadecanoic acid, linoleic ethyl ester, nonacosane, and squalene) were associated with the resistance mechanism of shallots against anthracnose. These secondary metabolites can be used as markers in selecting shallot genotypes resistant to anthracnose. However, further studies in different populations are necessary to understand the inheritance pattern of the four marker compounds of shallot resistance to anthracnose.

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