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HARNESSING METABOLOMICS TO COMBAT FUSARIUM WILT IN PIGEONPEA: A LC-MS AND GC-MS PERSPECTIVE

[1]Megha Ujinwal, [1]*Nisha Singh and [2]Sapna Langyan

[1]Gujarat Biotechnology University, Gujarat-382355, India

[2]ICAR-National Bureau of Plant genetic Resources, New Delhi, India 110012

*Corresponding Author

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ABSTRACT

Introduction

Pigeonpea has been a traditional staple in India, valued for its nutritional and therapeutic benefits since ancient times. Renowned for its diverse pharmacological properties, it exhibits antibacterial, anti-inflammatory, antitubercular, antioxidant, neuroprotective, antihypertensive, antihyperglycemic, and anticancer activities. However, its cultivation is challenged by various biotic and abiotic stress factors. Among them, Fusarium wilt (*Fusarium udum Butler*), a devastating soil-borne disease, causes significant yield losses across major pigeonpea production areas.

Objectives

This study aims to identify key metabolites involved in defense pathways and their interactions with the host plant by analyzing and comparing the metabolite profiles of healthy and diseased (fusarium wilt-infected) pigeonpea genotype UP 9-I using GC-MS and LC-MS/MS.

Methods

LC-MS and GC-MS untargeted metabolomics were used to explore and analyze pigeonpea genotype (UP 9-I). For LC-MS/MS, 0.25g of fresh leaves (healthy & diseased) were mixed with 30 mL water-methanol, filtered (0.2 µm), and 5 µL was injected for analysis. For GC-MS, 50 mg of leaves were crushed with 2 mL 80% methanol, concentrated (100 µL), derivatized with 100 µL MSTFA, incubated (65°C, 1 hour), filtered (0.2 µm), and injected. The extract was filtered (0.2 µm filter) and transferred for GC-MS analysis.

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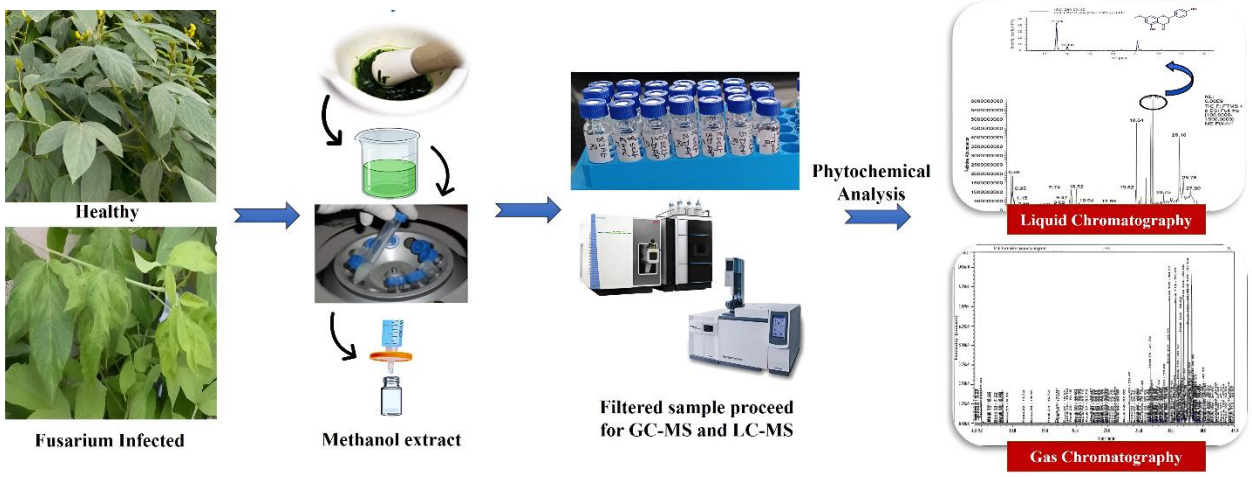
Results

Metabolomic analysis identified 152 metabolites in diseased plants, 159 metabolites in healthy plants, and 65 metabolites common to both healthy and diseased plants, including flavonoids, terpenoids, fatty acids, alkaloids, and amino acids. In addition, seven differential metabolic pathways (biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, steroid biosynthesis, cutin, suberine, and wax biosynthesis, fatty acid elongation, brassinosteroid biosynthesis, and fatty acid degradation) were elucidated. The LC-MS analysis identified the presence of Sakuranetin (an antimycobacterial agent), Apigetrin (a non-steroidal anti-inflammatory and antibacterial compound), Pinocembrin (an antineoplastic, vasodilatory, and neuroprotective agent), and Vitexin (a platelet aggregation inhibitor) in healthy pigeonpea leaves.

Conclusion

These compounds highlight the potential of pigeonpea extracts in addressing fungal pathogen-related disorders, providing insights into the plant's response to wilting stress. This study establishes a foundation for further research into the mechanisms of fusarium wilt resistance in pigeonpea, offering valuable information for plant breeding efforts.

Keywords: Enrichment, Fusarium wilt, Metabolites, Pathways, secondary metabolites.



Graphical abstract depicting the procedure of performing GC-MS and LC-MS of pigeonpea plant extract.

1. INTRODUCTION

Pigeonpea (*Cajanus cajan*), also referred to as red gram or arhar dal, originates from India and flourishes in tropical and subtropical regions worldwide (Sharma et al., 2016). It ranks as the sixth

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most important legume globally, accounting for 5% of total pulse production, covering 4.92 million hectares. India contributes over 70% of this area (3.6 million hectares) and boasts significant cultivar diversity, with 218 varieties comprising 73% of the total global (Jeevarathinam et al., 2020). Its significance lies in its adaptability to various agro-climatic conditions, from semi-arid to humid environments. This resilience has made pigeonpea a crucial component of food security and sustainable agriculture, particularly in regions vulnerable to drought and poor soil fertility. In addition to its agronomic importance, pigeonpea is highly valued for its nutritional benefits, serving as an excellent source of protein (20–22%), carbohydrates (65%), dietary fiber (3.8%), essential amino acids, macronutrients, and micronutrients, making it a key dietary component across various cultures (Singh et al., 2016; Kushwaha et al., 2023). Its role extends to enhancing soil health through nitrogen fixation, preventing erosion, and supporting crop rotation systems. Furthermore, pigeonpea plays a crucial role in intercropping strategies, promoting biodiversity and aiding in pest management. The fungus *Fusarium oxysporum* f. sp. *udum*, which causes fusarium wilt in pigeonpea, stands out as one of the most prominent and economically significant diseases. Fusarium wilt, a devastating disease in pigeonpea, causes 30–60% yield losses across major production regions by attacking the vascular system, leading to wilting, leaf yellowing, vascular discoloration, and ultimately plant death and reduced yields (Naik et al., 2022). First documented in India by Butler EJ. 1908, who conducted an extensive study on the pathogen, the disease presents a significant global challenge to pigeonpea production, particularly in warm and humid climates conducive to the fungus's growth (Jain et al., 1995; Patil et al., 2021). In India, fusarium wilt is particularly problematic in Karnataka, where the 2020–21 growing season saw the highest incidence in Kalaburagi (21.1%), followed by Raichur (16.67%) and Yadgir (7.7%), while Bidar recorded the lowest (3.5%). During the 2018–19 season, wilt severity across districts ranged from 3% to 13.7%, with localized areas experiencing the highest incidences (Naik et al., 2022). Wilted pigeonpea plants not only experience significant yield losses but also suffer from reduced seed quality, adversely affecting farmers' livelihoods and food security in affected regions. The lack of genetic resistance in many commercial pigeonpea cultivars to specific *fusarium* fungus strains further complicates disease management (Patil et al., 2021). Recent advancements in plant science have employed metabolomics, genomics, transcriptomics, and proteomics to evaluate plant performance under biotic stress. Among these, metabolomics is emerging as a critical tool for comparative analyses following genome and proteomics studies (Sayre-Chavez et al., 2022). Techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy have been used to study the effects of fungal infections. These findings highlight the potential of metabolomics, particularly when integrated with other technologies, to provide valuable insights into plant stress-response mechanisms (Heyneke et al., 2017; Luo et al., 2020; Joshi et al., 2021).

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This method analyzes primary and secondary metabolites, lipids, carbohydrates, organic acids, and amino acids simultaneously, revealing comprehensive insights into the metabolic status of plants.

Despite ongoing efforts to control fusarium wilt, its intricate interaction with pigeonpea remains poorly understood. This study aims to identify key metabolites involved in defense pathways and their interactions with the host plant by analyzing and comparing the metabolite profiles of healthy and diseased (fusarium wilt-infected) pigeonpea genotype UP 9-I using GC-MS and LC-MS/MS. By examining the metabolic changes in infected plants relative to healthy ones, the research seeks to uncover critical biochemical alterations triggered by pathogen attacks. The findings will enhance our understanding of disease-related metabolic shifts and contribute to developing effective strategies for disease management and crop improvement. Identifying metabolites linked to disease resistance can inform breeding programs aimed at cultivating fusarium wilt-resistant pigeonpea varieties. Furthermore, this study highlights essential metabolic processes that equip pigeonpea to combat Fusarium wilt and identifies metabolites that may improve resistance to wilting stress in breeding programs, including those for medicinal plants.

2. MATERIALS AND METHODS

2.1 Plant material and metabolite extraction

Fresh pigeonpea leaves of genotype UP 9-I (both fusarium wilt-infected diseased and healthy as a control) were collected during the flowering to pod-filling stage from fields at Gujarat Biotechnology University, Gandhinagar, Gujarat, India (Latitude 23.1684°N, Longitude 72.6743°E). The collected leaves were shade-dried to a consistent weight for extraction. The representation of healthy and diseased plant has shown in Fig. 1. For GC-MS analysis, 50 mg of both healthy and diseased leaves were crushed in a mortar and pestle with 2 ml of 80% methanol. The mixture was heated for one hour at 65°C in a water bath and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected into sterile microcentrifuge tubes and processed using a nitrogen evaporator to concentrate 100µl of the extract. To this, 100µl of Trimethylsilyl-N-methyl Trifluoroacetamide (MSTFA) solution was added. The samples were incubated on a heat block at 65°C for one hour, with the tubes inverted 2–3 times and vortexed every 10 minutes. Following incubation, 100µl of the sample was filtered using a sterile 0.2µm filter and syringe into an insert, ready for GC-MS analysis (Sosa et al., 2016; Anadebe et al., 2017).

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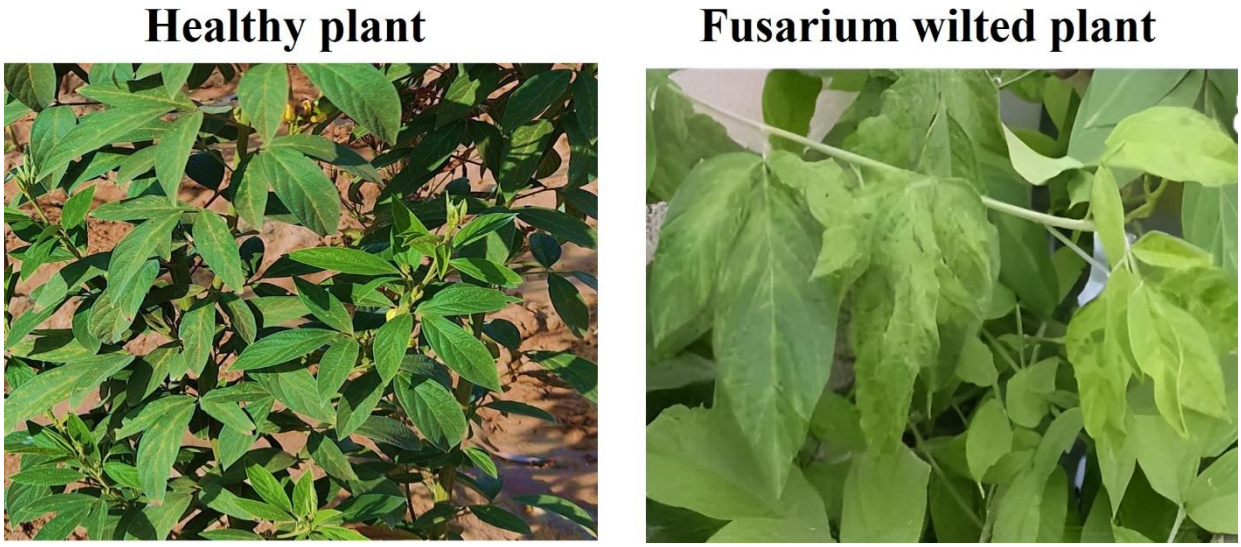

Fig. 1: Depiction of healthy and wilted pigeonpea plant at Gujarat Biotechnology University.

For LC-MS/MS analysis, fresh leaves from healthy and diseased pigeonpea plants were cleaned and cut into pieces before being ground using a mortar and pestle. A mixture of 0.25 g of leaves and 30ml of water with methanol solution was prepared. The mixture was sonicated for 5 minutes per sample and heated at 60°C for 15 minutes. After cooling, the sample was diluted to 50 ml and centrifuged at 5000 rpm for 5 minutes at 4°C. One milliliter of the supernatant was taken and centrifuged again at 10,000 rpm for 5 minutes at 4°C, followed by further dilution with methanol solution. The diluted samples were filtered through a 0.2µm syringe filter, and 5µl of the sample was transferred into the insert for LC-MS analysis (Ajaiyeoba et al., 2013; Wu et al., 2013).

2.2 LC-MS experimental system and measurements

All extracts (5µL injection volume) were analyzed in both positive and negative ionization modes (ESI+ and ESI−) using an Orbitrap Exploris 120 mass spectrometer coupled with a Vanquish autosampler from ThermoFisher (Massachusetts, USA). Two different chromatographic separations were employed: an ACQUITY UPLC HSS T3, 1.8µm, 100mm × 2.1 mm column, supplied by Waters (Massachusetts, USA), and an ACCUCORE 150 Amide HILIC, 2.6µm, 100mm × 2.1mm column, provided by Thermo Fisher Scientific (Massachusetts, USA), both of which were used in positive and negative ionization modes. The column temperature was maintained at a constant 35 °C in both ionization modes (Dinore et al., 2022).

2.3 GC-MS experimental system and measurements

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A Thermo Scientific TSQ-800 GC-MS system equipped with a TG-5-MS silica capillary column (30m × 0.25mm, film thickness 0.25µm) was used for compound analysis. The GC operation lasted 45 minutes, utilizing helium gas as the carrier at a flow rate of 1ml per minute. The oven temperature was initially maintained at 60°C for 2 minutes, then increased to 280°C at a rate of 5°C per minute and held isothermally at 280°C for 10 minutes. The ion source, detector, and injector port temperatures were set at 250°C, 260°C, and 280°C, respectively. The mass spectrometric detector operated in electron impact ionization mode, scanning a mass range of 50–700 m/z with a fragmentation energy of 70eV. Compound identification, including names, molecular weights, and structures, was performed using the National Institute of Standards and Technology (NIST) Library database (Dinore et al., 2022).

2.4 Qualitative, Pathway analysis and identification of secondary metabolites

To conduct a qualitative analysis of the samples, we manually checked for the presence of alkaloids, flavonoids, saponins, phenols, tannins, terpenoids, steroids, glycosides, phytosterols, and proteins. Secondary metabolites in the extracts of both healthy and diseased *Cajanus cajan* plants were identified based on LC retention time and high-resolution mass spectra. Pathway analysis was performed using MetaboAnalyst Version 6.0 (https://www.metaboanalyst.ca/), with the KEGG pathway database (https://www.genome.jp/kegg/pathway.html) serving as the reference for pathway examination. The analysis was carried out using the chemical names of all unique and common metabolites from both healthy and diseased pigeonpeas, which were submitted separately for enrichment analysis and metabolic pathway analysis (MetPA). Each metabolite was manually verified using KEGG and PubChem.

2.5 Statistical analysis

The obtained CSV file, including the normalized peak areas and identities (retention time (tr), mass-to-charge (*m/z*), molecular formula, and name) of the 311 metabolites identified in the three replicates of the two samples were exported to the online platform MetaboAnalyst Version 6.0 (https://www.metaboanalyst.ca/) for further chemometric analysis, including Hierarchical clustering heatmap (HCA), Volcano plot, principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA). Moreover, the variable importance in the projection (VIP) values was calculated to identify the metabolites that significantly differentiated between the two legume samples. Statistical differences between healthy and diseased groups were assessed using one-way ANOVA ($\alpha = 0.05$). Post-hoc corrections for multiple comparisons were not applied. Differential metabolites were considered significant at $p < 0.05$ and fold change ≥ 2. Furthermore, the biological significance of the identified metabolites was declared through the metabolite set enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Representation of the metabolic pathways is displayed according to their

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significance arranged by p-values (y-axis, pathway enrichment analysis) or pathway impact (x-axis, pathway topology analysis). The dot color is based on the p-value and its size is defined by the pathway impact values calculated from the matched metabolites.

3. RESULTS

3.1 Phytochemical characterization of pigeonpea leaf extracts using LC-MS

The LC-MS technique has become well-established and efficient, making it a more reliable option for the precise identification of secondary metabolites in plant material (Gopal et al., 2018; Wu et al., 2013). Using the LC-MS-ESI technique, 1021 compounds were identified in healthy plants, with 621 detected in the positive mode and 359 in the negative mode of ionization (Figure S2). The identified compounds include flavonoids, fatty acids, ketones, alkaloids, and glucosides. Among the primary metabolites, healthy plants consist of ketones, glucosides, methyl groups, and fatty acids. A total of 259 compounds were found to be common in both healthy and diseased plants (Fig. 2A).

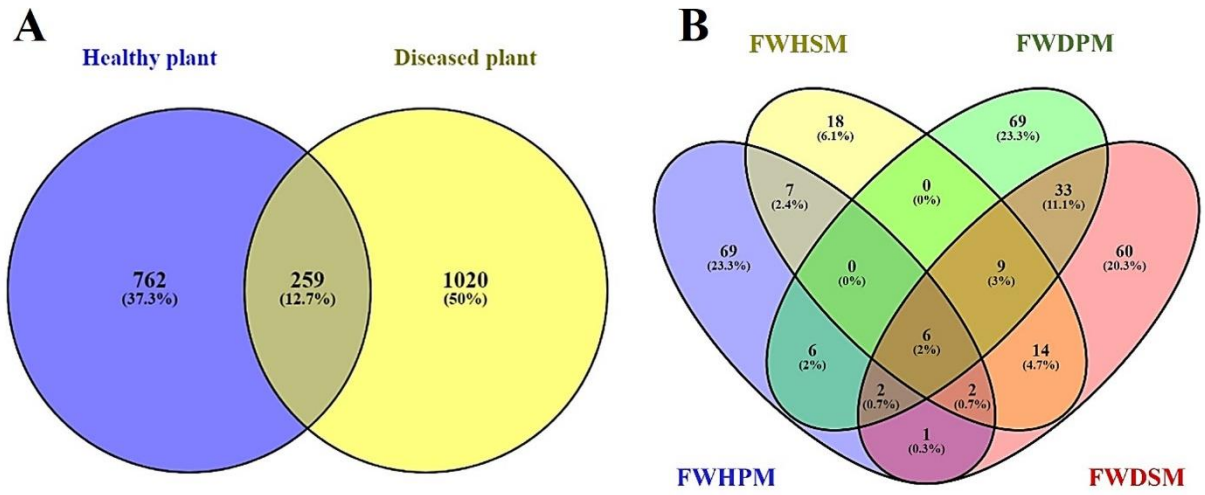

Fig. 2: (A) Representing the common number of metabolites in healthy and diseased plant and (B) Venn diagram depicting the common number of primary and secondary metabolites found common in both plants.

*FWHPM; Fusarium Wilt Healthy Primary Metabolite, FWHSM; Fusarium Wilt Healthy Secondary Metabolite, FWDPM; Fusarium Wilt Diseased Primary Metabolite, FWSPM; Fusarium Wilt Diseased Secondary Metabolite

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The diseased plant contained 1279 compounds, with 764 identified in the positive mode and 459 in the negative mode of ionization (Figure S3). The diseased plant exhibited higher levels of ketones, phenols, polysaccharides, nitriles, hydroxy groups, glucosides, alkanes, and glycosides. Fig 4B shows the overlap of primary and secondary metabolites common to both healthy and diseased plants. Notably, the diseased plant had higher levels of flavonoid compounds compared to the healthy plants, followed by chalcones, polyphenols, and anthraquinones. LC-MS-ESI also identified compounds such as Apigetrin (a non-steroidal anti-inflammatory drug, metabolite, and antibacterial agent), Sakuranetin (an antimycobacterial drug), Pinocembrin (an antineoplastic, vasodilator, and neuroprotective agent), and Vitexin (a platelet aggregation inhibitor) in healthy pigeonpea plants. In diseased plants, compounds such as Chrysin (a flavonoid with anti-inflammatory, antineoplastic, antioxidant, and hepatoprotective properties), Capsidiol (a terpenoid with antifungal activity), Luteolin (a free radical scavenger, anti-inflammatory, immune system modulator, and anti-cancer agent), and Hispidulin (an apoptosis inducer, anti-inflammatory, and anticonvulsant agent) were identified. The spectral peaks and molecular structures of these identified metabolites are shown in Figures S4 and S5.

3.2 Metabolic profiles comparison and differentiating metabolites analysis

A volcano plot (Fig. 3) was used to obtain more insight into the metabolic differences between these clusters and to determine the differently accumulated compounds. Metabolites were considered differentially accumulated if they exhibited an absolute $\log_2$ fold change ≥ 1 (equivalent to FC ≥ 2 or ≤ 0.5) and a $-\log_{10}$(p-value) ≥ 1.3 (corresponding to $p < 0.05$). Of the 117 identified differential metabolites, 11 were statistically significant (4 upregulated, 7 downregulated; $p < 0.05$, FC ≥ 2), while 106 metabolites showed non-significant variation ($p > 0.05$).

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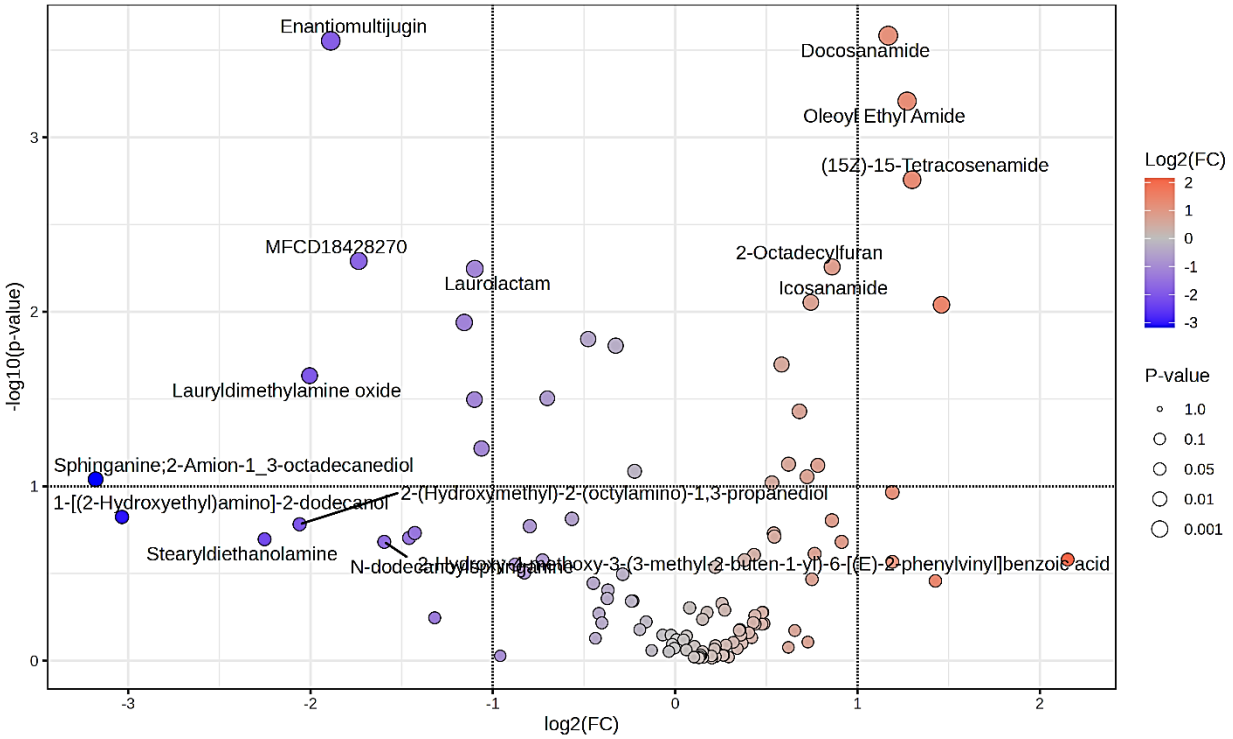

Fig. 3: Volcano plot of differential metabolites of healthy and diseased pigeonpea plant. The fold-change value (FC) for each differential metabolite was transformed as Log2, and the corresponding p-value was transformed as − Log10.

The plot effectively highlights key metabolites that are differentially expressed, which may be of biological importance. Consequentially, the discriminating metabolites colored red were substantially higher in healthy while those colored blue were significantly higher in diseased plant. Docosanamide, Oleoyl Ethyl Amide, (15Z)-15-Tetracosenamide, Icosanamide were found more significant upregulated metabolites having positive log2(FC) value (Red coloured). While Enantiomultijugin, Sphinganine; 2-Amion-1,3-octadecanediol, Lauryldimethylamine oxide, Stearyldiethanolamine were found downregulated metabolites downregulated in the diseased group compared to the healthy. These all metabolites mainly comprised flavonoids, isoflavonoids, fatty acid, ketone and Terpenoid.

Further, PCA modeling was performed to provide a general visual separation of all of the samples. At 95% confidence limit, two principal components described the positions of the distinct metabolome clusters with PC1/PC2 accounting for 81.1% of the variance in metabolic profiles of the analyzed extracts. In the PCA scores plot (Fig. 4). Each condition (healthy and diseased pigeonpea leaves) was analyzed using three biological replicates (n = 3), ensuring statistical robustness and reproducibility . The results were similar to the HCA model, with the six samples

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grouped into two distinct areas in the plot and indicate a milder disease state or a sample with characteristics partially similar to healthy ones.

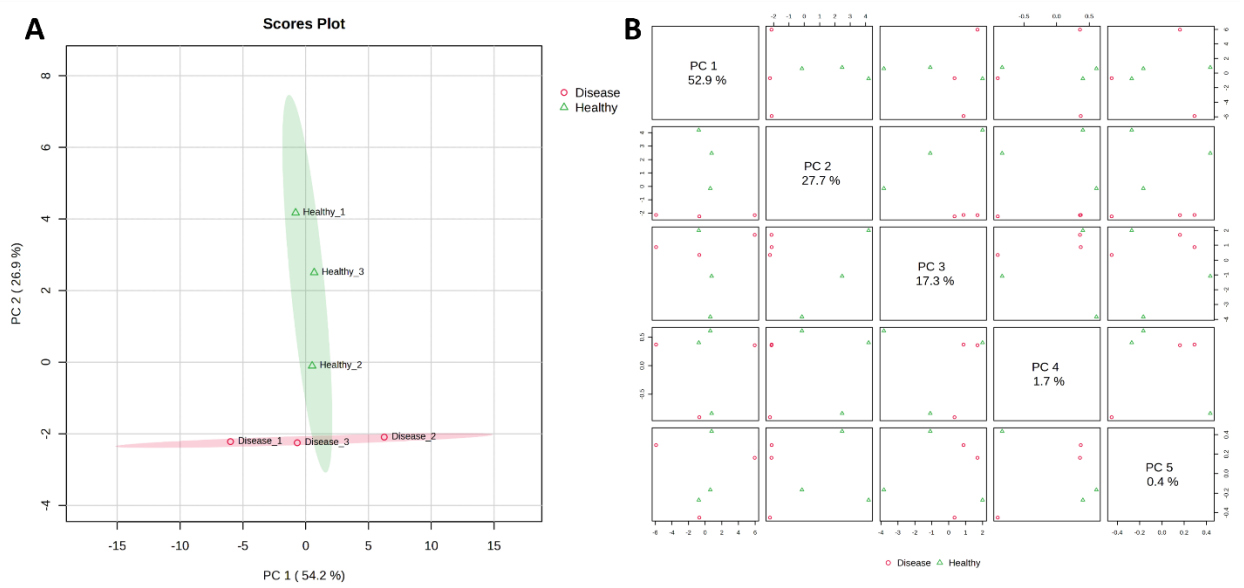

Fig. 4: A. Principal component analysis (PCA) scores plot using the identified metabolites by LC/MS analysis of the six pigeonpea samples. B. PCA overview.

Unlike PCA, PLS-DA is a supervised multivariate analysis method that can maximize the differences between different groups by using partial least squares regression to model the relationship between metabolite expression and sample class to achieve modeling prediction of the studied samples. High predictability (Q^2) and strong goodness of fit (R^2X, R^2Y) of the PLS-DA model were observed ($Q^2 = 0.91$, $R^2X = 0.94$, $R^2Y = 0.96$). These results revealed great predictability and goodness of fit of the constructed PLS-DA model. As can be observed in the PLS-DA VIP plot (Fig. 5), the variable importance in the projection (VIP) value of the first principal component of the PLS-DA model was used at $p < 0.05$ to find the unique compound for each sample.

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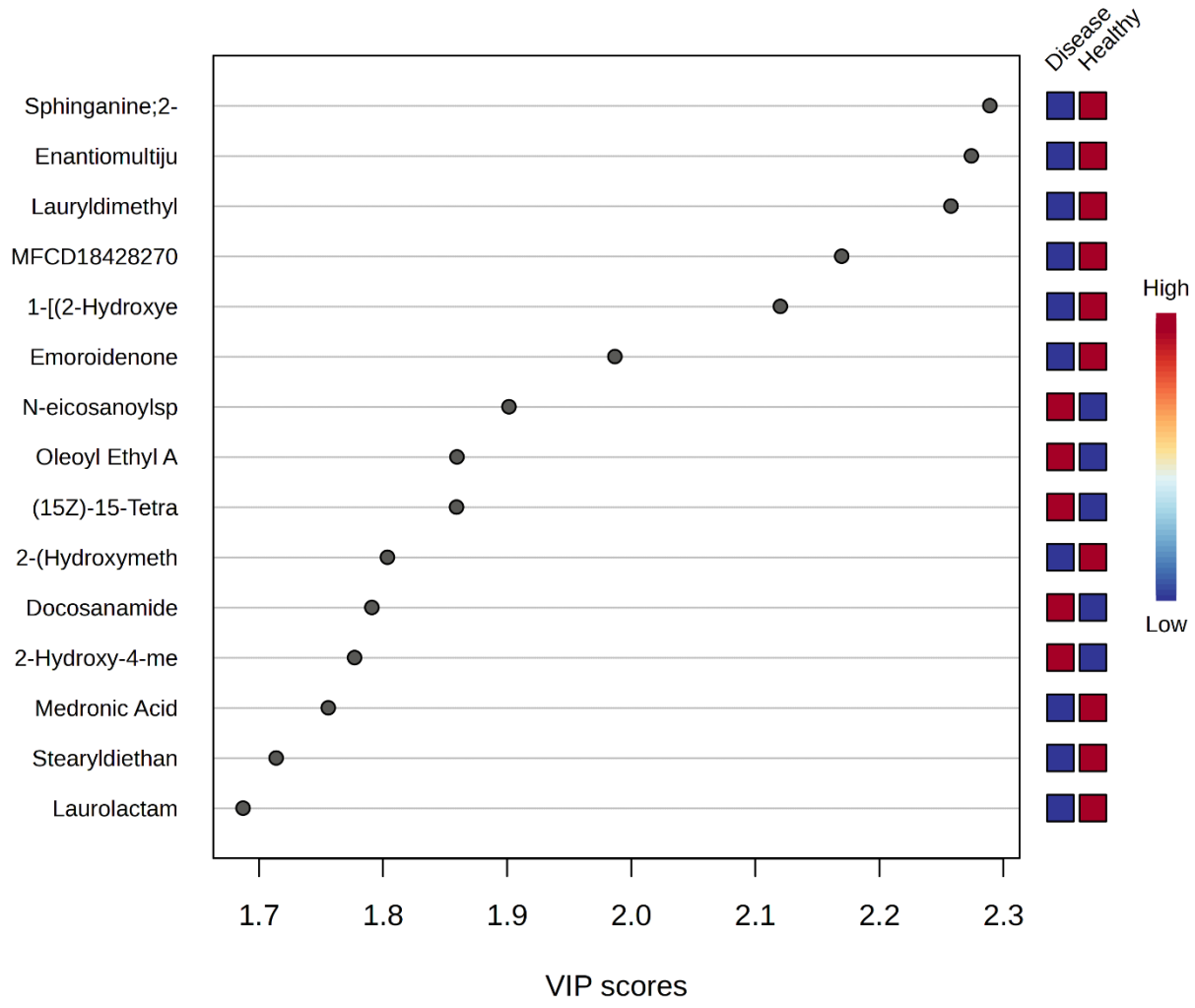

Fig. 5: Partial least squares discriminant analysis (PLS-DA) the variable importance in projection (VIP) score showing the top 15 differential metabolites (VIP scores > 1) in the methanolic extracts of the healthy and diseased analyzed samples.

3.3 GC-MS analysis of untargeted metabolites in diseased and healthy sample

GC-MS remains an essential method for analyzing non-polar and volatile compounds such as essential oils, fatty acids, lipids, esters, and alkaloids in plants (Sosa et al., 2016). A total of 65 common compounds were identified in both healthy and diseased plants. The healthy samples contained 94 unique compounds, including three fatty acids, two terpenes, two sesquiterpenoids, two flavonoids, one terpenoid, one dimethoxy flavanone, one tocotrienol, one monoterpene, and two sterols (Table S1). Notably, the diseased plant significantly altered the secondary metabolite

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profile of pigeonpea, leading to the identification of 87 compounds, unique to diseased sample only. These included two sesquiterpenoids, two fatty acids, one polyphenol, one terpene, and one diterpene, which were induced in response to the diseased state (Table S2). The GC-MS chromatogram of the healthy and diseased plants (Figure S1) revealed a total of 8 and 14 probable phytocompounds, respectively, with retention times ranging from 4.02 min to 35.75 min for healthy plants and from 4.18 min to 44.12 min for diseased plants. These compounds are tabulated in Table 1 and Table 2.

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Table 1: Detail information of identified metabolites in healthy pigeonpea leaves samples using GC-MS analysis.

Compound Name	RT	Molecular mass (g/mol)	Molecular formula	Peak area	Compound Nature	Annotation
4H-1-Benzopyran-4-one, 2,3-dihydro-5-hydroxy-7-methoxy-2-phenyl-, (S)-	35.75	270.28	$C_{16}H_{14}O_4$	16.41	Flavonoid	It has a role as an antidote. Antidote are medical compounds in response to poisonings. These compounds are secondary metabolites which can reduce the toxic effect of venom.
Farnesol, TMS derivative	27.62	294.5	$C_{18}H_{34}OSi$	0.08	sesquiterpenoid	It has a role as a fungal metabolite and an antimicrobial agent. It is a farnesane sesquiterpenoid, a primary alcohol and a polyprenol.
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	32.82	278.4	$C_{18}H_{30}O_2$	0.28	Flavonoid	It has a role as an antithrombotic effect. It has a role as a micronutrient, a nutraceutical metabolite.
2,4-Di-tert-butylphenol	20.48	206.32	$C_{14}H_{22}O$	0.04	Phenolic	It has a role as a bacterial metabolite, an antioxidant and a marine metabolite. It is an alkylbenzene and a member of phenols.

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Acetamide, 2,2,2-trifluoro-N-methyl-	4.02	127.07	$C_3H_4F_3NO$	0.03	sesquiterpenoid	It has a role as a chromatographic reagent. It is a monocarboxylic acid amide, a N-silyl compound and a trifluoroacetamide.
1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	21.73	222.37	$C_{15}H_{26}O$	0.05	sesquiterpenoid	It has a role as a flavoring agent, a cosmetic, a pheromone, a neuroprotective agent, an antifungal agent, an anti-inflammatory agent, an antihypertensive agent, an antioxidant, a volatile oil component, an insect attractant and an herbicide.
Caryophyllene	18.29	204.35	$C_{15}H_{24}$	0.12	sesquiterpenoid	It has a role as a non-steroidal anti-inflammatory drug, a fragrance, a metabolite and an insect attractant.
Tetradecanoic acid	26.27	228.37	$C_{14}H_{28}O_2$	0.43	Flavonoid	It is a long-chain fatty acid and a straight-chain saturated fatty acid.

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Table 2: Detail information of identified metabolites in diseased pigeonpea leaves samples by GC-MS analysis.

Compound	RT	Molecular mass (g/mol)	Molecular formula	Peak area	Compound Nature	Annotation
Acetamide, 2,2,2-trifluoro-N-methyl-	4.19	127.07	$C_3H_4F_3NO$	0.01	Amide	It has a role as a chromatographic reagent.
Benzaldehyde	6.34	106.12	C_7H_6O	0.04	Benzene aldehyde	It has a role as a flavoring agent, a fragrance, an odorant receptor agonist, an EC 3.5.5.1 (nitrilase) inhibitor and an EC 3.1.1.3 (triacylglycerol lipase) inhibitor.
Pentanoic acid, TMS derivative	6.46	174.31	$C_8H_{18}O_2Si$	0.09	Fatty acid	It has a role as a plant metabolite. It is a short-chain fatty acid and a straight-chain saturated fatty acid.
Cyclohexane, isocyanato-	6.73	125.17	$C_7H_{11}NO$	0.04	Alkane	Used to make pharmaceuticals and agricultural chemicals.
Piperidine	12.74	85.15	$C_5H_{11}N$	0.15	Flavonoid	It has a role as a reagent, a protic solvent, a base, a catalyst and a non-polar

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						solvent. It is a saturated organic heteromonocyclic parent, an azacycloalkane, a secondary amine and a member of piperidines.
Tetrachloroisophthalonitrile	27.83	265.9	$C_8Cl_4N_2$	0.11	Nitrile	It has a role as an antifungal agrochemical.
Neophytadiene	28.18	278.5	$C_{20}H_{38}$	0.10	Diterpene	It has a role as an anti-inflammatory agent, an antimicrobial agent, a plant metabolite and an algal metabolite. It is an alkene and a diterpene.
n-Hexadecanoic acid	30.57	256.42	$C_{16}H_{32}O_2$	1.26	fatty acid	It is a long-chain fatty acid and a straight-chain saturated fatty acid.
Phytol	32.51	296.5	$C_{20}H_{40}O$	0.12	Terpenoid	It is a diterpenoid and a long-chain primary fatty alcohol.
4H-1-Benzopyran-4-one, 2,3-dihydro-5-hydroxy-7-methoxy-2-phenyl-, (S)-	35.69	270.28	$C_{16}H_{14}O_4$	10.73	Flavonoid	It has a role as a plant metabolite and an antidote.
4H-1-Benzopyran-4-one, 2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-, (S)-	38.67	286.28	$C_{16}H_{14}O_5$	0.15	Flavonoid	It has a role as an antimycobacterial drug and a plant metabolite.

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?-Tocopherol	40.83	430.7	$C_{29}H_{50}O_2$	0.07	phytosterols	It has a role as an antioxidant, a nutraceutical, an antiatherogenic agent
dl-a-Tocopherol	41.64	430.7	$C_{29}H_{50}O_2$	0.26	phytosterols	It has a role as an antioxidant, a nutraceutical, an antiatherogenic agent, an EC 2.7.11.13 (protein kinase C) inhibitor, an anticoagulant, an immunomodulator, an antiviral agent, a micronutrient, an algal metabolite.
?-Sitosterol	44.13	414.7	$C_{29}H_{50}O$	0.28	phytosterols	It has a role as a sterol methyltransferase inhibitor, an anti-cholesteremic drug, an antioxidant.

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GC-MS analysis of both extracts revealed the presence of key phytoconstituents, including flavonoids (40%), sesquiterpenes (25%), and fatty acids and phenols (15%). In healthy pigeonpeas, 29 esters, 25 alcohols, 24 ketones, and 23 phenols were identified. In contrast, the diseased pigeonpea plant contained 37 alcohols, 19 ketones, 21 esters, and 14 carboxylic acids. These identified compounds are associated with antithrombotic effects, serve as micronutrients, and are considered nutraceutical metabolites. Additionally, the compound listed in Table 2 were identified as flavoring agents and protic solvents. Healthy pigeonpea sample also contained sesquiterpenoid which are annotate for anti-microbial, neuroprotective, antifungal, anti-inflammatory, antihypertensive, antioxidant, and insect-attractant properties. These compounds also serve as non-steroidal anti-inflammatory drugs, fragrances, metabolites, and insect attractants. Acetamide, 2,2,2-trifluoro-N-methyl, a chromatographic reagent, and dl-α-tocopherol, which functions as an antioxidant, antiatherogenic agent, anticoagulant, immunomodulator, antiviral agent, micronutrient, and algal metabolite, were commonly identified metabolites in both samples. Interestingly, the diseased plant sample also consist significant compounds annotate for antimycobacterial properties, potentially useful for treating chronic diseases like tuberculosis and leprosy. The diseased plants notably altered the secondary metabolite profile of pigeonpea, leading to the production of 87 compounds unique to diseased plant sample only. Among these, two sesquiterpenoids, two fatty acids, one polyphenol, one terpene, and one diterpene were identified as responses to the disease (Table S2). GC-MS analysis of healthy and diseased plants (Figure S1) revealed 8 and 14 phytocompounds, respectively, with retention times ranging from 4.02 min to 35.75 min for the healthy plant and 4.18 min to 44.12 min for the diseased plant, as shown in Tables 1 and 2.

3.4 Metabolic Pathway Analyzed by GC-MS in healthy and disease pigeonpea plant

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to link the identified metabolites to their metabolic pathways. Metabolite set enrichment analysis (MSEA) was utilized to classify the chemical groups of all identified compounds and highlight the most enriched metabolic pathway in the analyzed healthy and fusarium-wilt diseased samples. The enrichment bubble diagram represents the chemical classifications of the enriched metabolite sets. Pathway analysis, based on enrichment procedures, identifies the most relevant metabolic pathways by assessing pathway impact and ranking them according to the lowest log10(p) value. Both healthy and diseased plants activated eight metabolic pathways (Tables S3 and S4). Among these differentially enriched metabolic pathways, seven had impact values >0.1, which is the correlation threshold following pathway enrichment and topological analysis. Based on negative log (*p*) and impact values, we identified significantly correlated pathway in both healthy and diseased plant for biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, steroid biosynthesis, cutin, suberine, and wax biosynthesis, fatty acid elongation, brassinosteroid biosynthesis, and fatty acid

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degradation (Fig. 5). However, sesquiterpenoid and triterpenoid biosynthesis ($p < 0.05$) were unique to healthy plants, while phenylpropanoid biosynthesis ($p > 0.05$) was specific to diseased plants. In healthy plants, fatty acid biosynthesis exhibited the lowest log(p) value (0.003), whereas in diseased plants, the biosynthesis of unsaturated fatty acids had the lowest log10(p) value (0.01) (Tables S3 and S4).

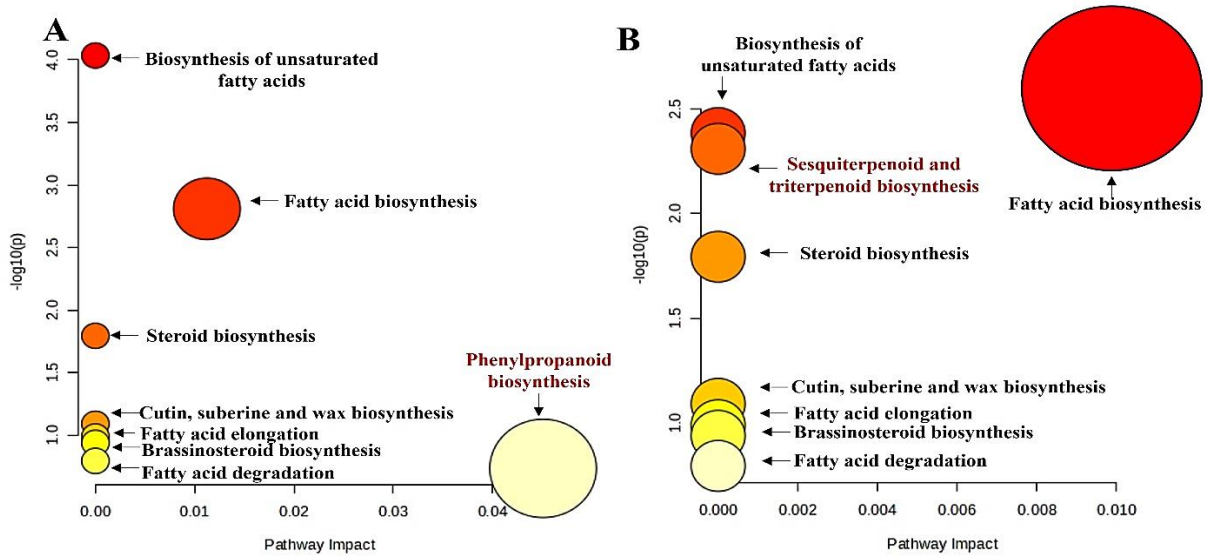

Fig. 5: Metabolic pathways analysed in healthy (A) and diseased (B) in pigeonpea plant. Each bubble in the figure is a metabolic pathway, with the x-axis indicating pathway enrichment and the y-axis indicating pathway impact. The size and color represent the enrichment and impact values of the major pathway, respectively.

Both plants share hexadecanoic acid, a long-chain fatty acid, as the most common compound across these 8 metabolic pathways. Humulene and beta-caryophyllene are found in the sesquiterpenoid and triterpenoid biosynthesis pathways, while coniferyl alcohol is present in the phenylpropanoid biosynthesis pathway (Tables S3 and S4).

3.5 Metabolic pathway enrichment analysis in healthy and diseased pigeonpea plants

Metabolite set enrichment analysis reveals biologically significant patterns that are significantly enriched in quantitative metabolomic data. In our study, nine metabolites in healthy plants were found to be enriched in eight pathways: fatty acid biosynthesis, glycerolipid metabolism, plasmalogen metabolism, mitochondrial beta-oxidation of long-chain saturated fatty acids, fatty acid elongation in mitochondria, fatty acid metabolism, steroid biosynthesis, and bile acid biosynthesis (Table S5). These enriched pathways highlight palmitic acid as the most common metabolite.

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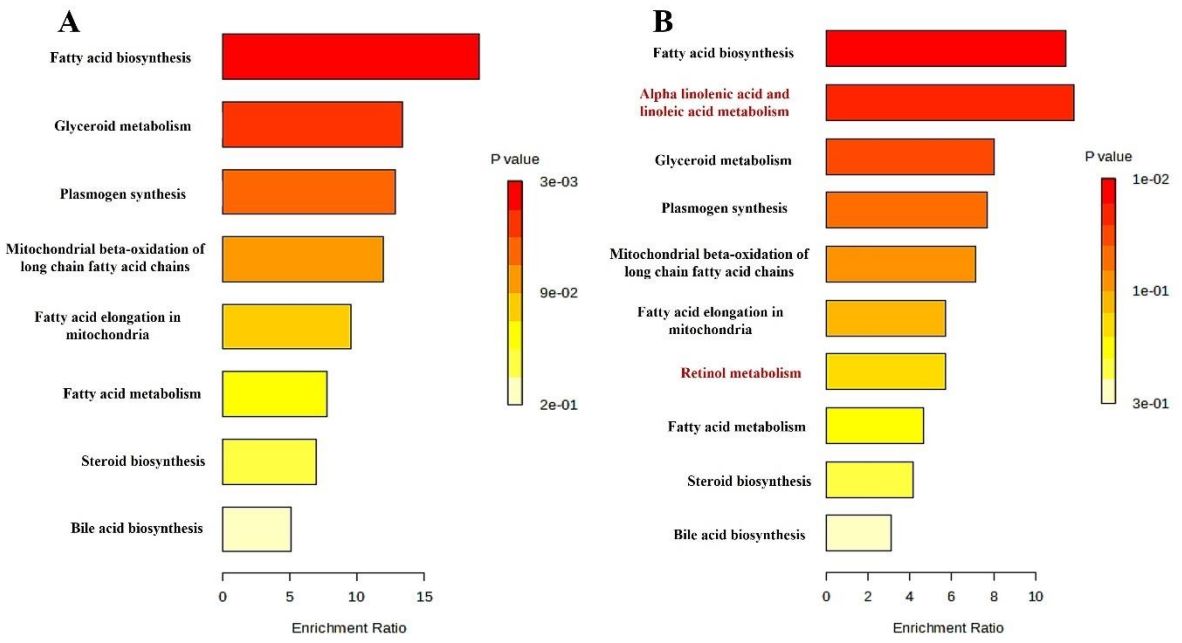

Fig. 6: Metabolite set enrichment analysis pathway in healthy (A) and diseased (B) in pigeonpea plant. The horizontal coordinate indicates the ratio of the differential metabolite numbers in the corresponding pathway to the total identified metabolite numbers in this pathway, and the larger the ratio value, the greater the enrichment of this pathway, and the vertical coordinate indicates the name of the pathway. The color intensity reflects the statistical significance of the identified pathways, the darker the color, the more affected the pathway.

In diseased plants, 11 metabolites are prominently enriched in 10 pathways, including fatty acid biosynthesis, alpha-linolenic acid and linoleic acid metabolism, glycerolipid metabolism, plasmalogen synthesis, mitochondrial beta-oxidation of long-chain saturated fatty acids, fatty acid elongation in mitochondria, retinol metabolism, fatty acid metabolism, steroid biosynthesis, and bile acid biosynthesis (Table S6). Among these 11 metabolites, palmitic and stearic acids are the most common. The fatty acid biosynthesis pathway shows the lowest p-value (<0.05) in both healthy and diseased pigeonpea plants (Fig. 6).

3.6 Analysis of associations between metabolic changes

A Pearson's correlation analysis was performed to evaluate the relationship between the healthy and diseased annotated metabolites. Only the correlations with $P < 0.05$ were highlighted in the heat map. It has been observed in Fig. 7, certain relationships between the abundance of the identified metabolites in the six extracts and the bioactivity with Pearson's correlation coefficient (r) $r > \pm 0.5$ at $p < 0.05$ and false discovery rate (FDR) < 0.01.

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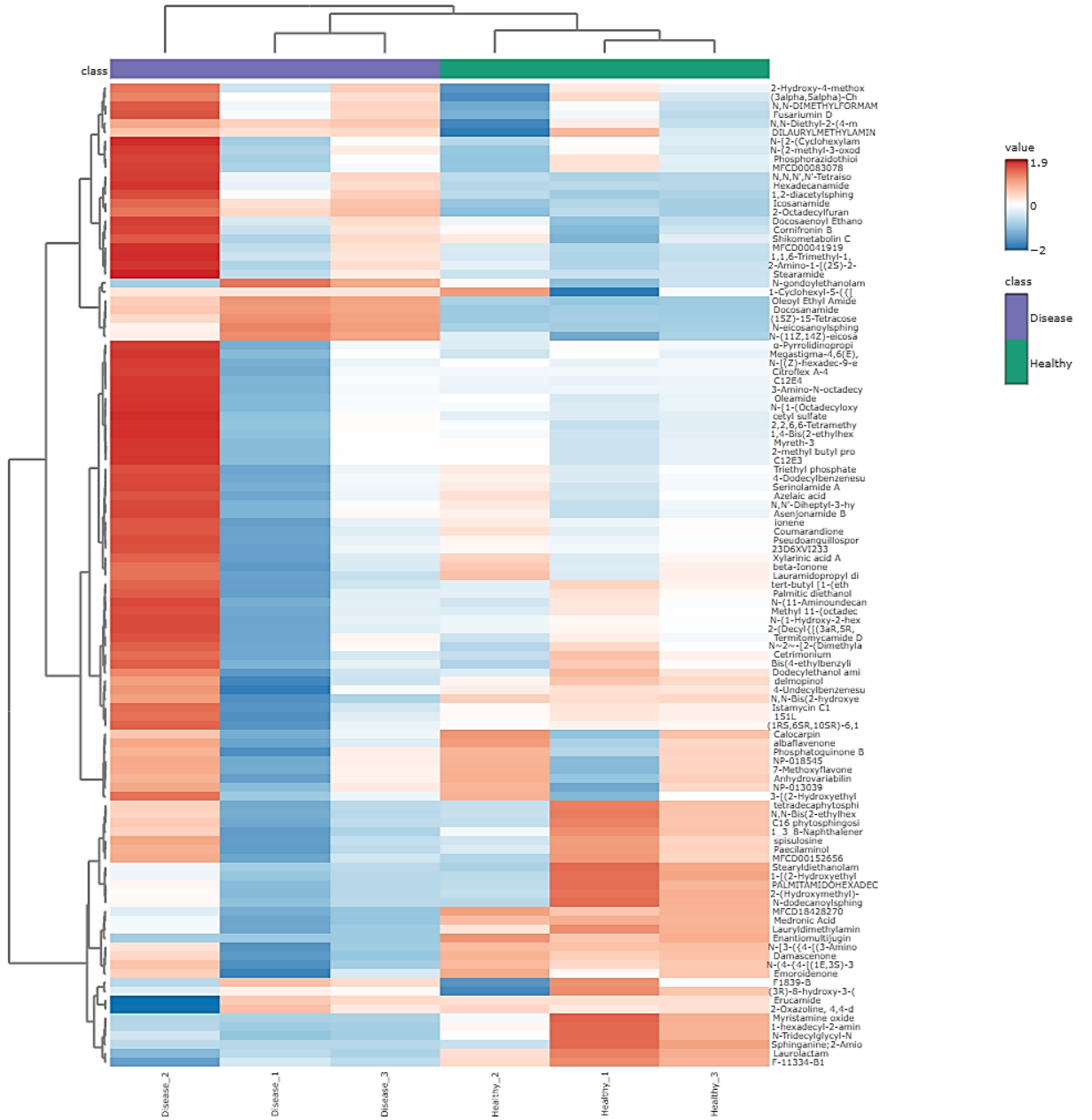

Fig. 7: The absolute values of Pearson correlation coefficients heatmap above the threshold are shown (*P*<0.01), and the correlation levels are indicated as red (positive correlation) or blue (negative correlation), with darker colors indicating stronger correlations.

Indeed, 97 metabolites were positively correlated includes flavonoids (N,N,N',N'-Tetraisobutyl-1,2-cyclohexanedicarboxamide, Laurolactam, Bis(4-ethylbenzylidene)sorbitol and Anhydrovariabilin), terpenoid (N-(2-methyl-3-oxodecanoyl)pyrrolidine, Xylarinic acid A), fatty

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acid derivatives. In contrast, only 20 metabolites exhibited a negative correlation with the activity and were mainly steroids, phenolic derivatives and anthraquinone. To further validate these findings a docking study was performed to explore the potential binding modes and the intermolecular interactions of the detected compounds.

4. DISCUSSION

In pigeonpea cultivation, *Fusarium udum* induced wilt disease is a significant biotic factor that negatively impacts crop growth and yield, with disease incidence ranging from 30–60% during the flowering and maturity stages (Sosa et al., 2016). Managing fusarium infection is challenging due to its soil-borne nature, deep penetration into host tissues, synthesis of long-lasting dormant structures, and ability to survive for extended periods without a host plant (Boukerma et al., 2017; Wang et al., 2018). A literature review revealed that limited studies have focused on GC-MS and LC-MS investigations of pigeonpea leaf phytoconstituents. To the best of our knowledge, this study is the first to explore the metabolome of *Cajanus cajan*, a medicinal and food plant, in relation to its resistance to fusarium wilt. Using both GC-MS and LC-MS techniques, we examined the phytoconstituents present in the leaves of healthy and diseased pigeonpea plants, assessing their chemical composition and functional roles. Notably, ketones, alcohols, and esters predominated in both healthy and diseased plants. The high prevalence of alcohols and esters in the leaf extracts underscores their importance in the plant's metabolic response to stress. These compounds are known to accumulate under various stress conditions, acting as signaling molecules that initiate adaptive responses (Eljounaidi et al., 2016; Butkutė et al., 2018). The GC-MS analysis also identified fatty acids, such as tetradecanoic acid, in healthy pigeonpea leaves, which are recognized for their medicinal value in phytomedicine (Anadebe et al., 2017). The concentration study showed that metabolites were present in higher levels in diseased plants compared to healthy ones, suggesting that the plant's defense mechanisms are directly correlated with the concentration of secondary metabolites or active compounds in the extracts. Elevated secondary metabolite levels in diseased (fusarium-infected) plants indicate that these compounds are being produced in greater quantities to combat fungal activity (Basaar et al., 2017; Manzo et al., 2017). Additionally, increased concentrations of secondary metabolites enhance plant resistance; however, these metabolites may also exhibit phytotoxic effects, potentially contributing to the pathogenicity of *Fusarium udum* in pigeonpea plants (Singh et al., 2015; Goel et al., 2013). The observed rise in secondary metabolites in diseased plants aligns with findings suggesting the suppression of plant immune responses during pathogen attacks (Hossain et al., 2024; Sattiraju et al., 2019). Our study identified a wide range of phytochemicals, including flavonoids, polyphenols, fatty acids, and amides, involved in the fatty acid biosynthesis pathway as part of the plant's response to fungal stress. The presence of metabolites like sesquiterpenoids, fatty acids, polyphenols, terpenes, and diterpenes in diseased plants further supports the idea of a complex interaction between plant-

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pathogen dynamics and metabolic responses (Liu et al., 2022; Patil et al., 2021). Previous studies reported that the major metabolic pathways in legumes included flavone and flavonol biosynthesis, aminoacyl-tRNA biosynthesis, isoquinoline alkaloid biosynthesis, the biosynthesis of amino acids, and isoflavonoid biosynthesis which is in accordance with our results (Shi et al., 2022).

The GC-MS analysis revealed the presence of tetradecanoic acid, caryophyllene, and farnesol in diseased pigeonpea plants. Furthermore, the LC-MS study identified chrysin and hispidulin, which are known for their antioxidant and anticonvulsant properties, in diseased plants, while apigetrin and pinocembrin, recognized for their anti-inflammatory and neuroprotective effects, were identified in healthy plants. Notably, these compounds were not detected in other legumes. Both LC-MS and GC-MS analyses showed that pigeonpea leaves are a rich source of flavonoids, terpenoids, essential oils, and coumarins, which have potential as medicinal agents due to their analgesic and antibacterial properties (Anadebe et al., 2017). Previous metabolomic studies have identified a variety of compounds in other legumes, such as lipids, sugars, amino acids, and amines in mung bean seeds, faba bean, and a diverse range of compounds in soybean sprouts (Gu et al., 2017; Ferreres et al., 2017; Khan et al., 2019). We conducted pathway analysis on both diseased and healthy pigeonpea plants to explore the metabolic pathways involved in the response to fusarium infection. This analysis provided valuable insights into the dynamic changes in metabolite concentrations and pathway activities, consistent with earlier studies that emphasize the significance of pathway analysis in understanding plant-pathogen interactions and metabolic responses (Sattirajuet al., 2019). General defense mechanisms include the production of antifungal proteins and the activation of defense signaling pathways, while pathogen-specific mechanisms involve the recognition of specific pathogen effectors by plant resistance gene products and the detoxification of pathogen-specific toxins (Liu et al., 2022). In line with previous research, the increased abundance of certain secondary metabolites, such as fatty acids and terpenoids, in diseased plants suggests their role in plant defense mechanisms against fusarium wilt. These metabolites may act as signaling molecules or antimicrobial agents to reduce pathogen-induced damage, as supported by the existing literature on plant defense responses (Powell et al., 2017; Rana et al., 2017). The activation of pathways related to steroid biosynthesis, monoterpenoid biosynthesis, and fatty acid biosynthesis indicates that the plant is enhancing its defense mechanisms and stress resilience in response to fusarium wilt (Kankanala et al., 2019). Other studies have highlighted the role of fatty acid content and related pathways in immune and stress responses in bean plants. This study also explored the biological regulatory mechanisms of newly discovered rhizobacteria and their roles in disease suppression and promoting plant growth in pigeonpea. These rhizobacteria produce biocidal chemicals and antifungal metabolites to combat fungi (Dukare et al., 2021; Du et al., 2022). Additionally, the LC/GC-MS analysis identified the brassinosteroid (BRs) pathway in both healthy and diseased plants. BRs, a group of plant steroidal hormones, regulate developmental processes and are crucial for managing stress (Kour et al., 2021;

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Choudhary et al., 2020). In this context, potential metabolites identified in metabolic pathways might serve as therapeutic targets and contribute to the development of broad-spectrum drugs. However, to ensure transparency in metabolomic research, it is essential to recognize the inherent analytical limitations of the LC-MS and GC-MS techniques that we used in this study. The study highlights diverse classes of metabolites—flavonoids, alkaloids, fatty acids, terpenoids, phenolics—present in both healthy and diseased pigeonpea leaves. Such diversity complicates ionization efficiency and quantification accuracy due to matrix effects (signal suppression or enhancement). For instance, the higher presence of ketones, alcohols, and esters in diseased samples might lead to differential ion suppression, making accurate quantification challenging. Another limitation is derivatization dependency, we used MSTFA derivatization for GC-MS analysis to volatilize polar compounds. However, derivatization can be inconsistent, affecting reproducibility, especially when comparing metabolite levels across treatments (healthy vs. diseased). Some compounds may be incompletely derivatized or degrade at the high GC inlet temperatures, limiting detection. Moreover, the study reports 159 metabolites in healthy and 152 in diseased pigeonpea via metabolite set enrichment, but not all compounds were unambiguously identified. With all these limitations, this study successfully utilized GC-MS and LC-MS to differentiate metabolic responses of pigeonpea to Fusarium wilt, acknowledging the above limitations is critical for reproducibility and transparency. These include challenges in compound coverage, derivatization efficiency, database reliance, quantification accuracy, and sample matrix effects. Addressing these will enhance the reliability of future metabolomics studies, especially when translating findings into breeding or therapeutic applications. It can aid in the development of testable predictions, the understanding of drug action mechanisms, and the increase of research productivity towards novel drug discovery. This study contributes to the development of novel natural drugs with high potency and low toxicity derived from pigeonpea leaves.

5. CONCLUSION

This comprehensive study elucidates the complex metabolic alterations and key biosynthetic pathways associated with fusarium wilt, a fungal infection in pigeonpea plants. Through extensive metabolite research of both healthy and Fusarium-infected UP 9-I pigeonpea plants, we gained new insights into the biochemical changes induced by the pathogen. The metabolism of fusarium infection is intricate, by the identification of 159 and 142 metabolites in healthy and diseased (Fusarium-infected) plants, respectively. Esters and alcohols were more prevalent in both healthy and diseased pigeonpea plants. The increased levels of secondary metabolites in diseased plants act as antimicrobial agents or signalling molecules to mitigate pathogen-induced damage. Healthy plants exhibited a higher baseline level of certain secondary metabolites, potentially functioning as a défense mechanism. This comparative analysis using GC-MS and LC-MS/MS highlights the complex metabolic responses of pigeonpea to fusarium wilt. The findings emphasize the

importance of secondary metabolites in plant défense and provide a foundation for developing disease-resistant pigeonpea varieties. Further research is required to explore the specific roles of the identified metabolites in disease resistance and their potential applications in breeding programs.

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Data Availability

The datasets produced and/or analyzed in the course of the present study can be obtained from the corresponding author on reasonable request.

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