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RESEARCH ARTICLE

# Comparative metabolic profiling of resistant and susceptible mungbean (*Vigna radiata* L. Wilczek) genotypes to elucidate the defense response against mungbean yellow mosaic virus (MYMV) disease

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## Abstract

Mungbean Yellow Mosaic Virus (MYMV) disease significantly impacts mungbean crop productivity, with the losses ranging from 10 to 100 percent. Developing host plant resistance offers a sustainable solution to mitigate this challenge. The metabolic changes underlying resistance to MYMV remain primarily unexplored in mungbean. The present study used nontargeted metabolomic profiling to analyze the comparative metabolic changes in resistant and susceptible genotypes upon disease incidence. The methanol extract of leaf samples collected from MYMV disease resistant (GAM 5) and susceptible (ADT 3) genotypes upon occurrence of MYMV disease were subjected to gas chromatography - mass spectrophotometry (GC-MS) analysis. Metabolic profiling resulted in the identification of 40 and 49 metabolites in resistant and susceptible genotypes, respectively. The fold change analysis revealed that 12 metabolites showed significant differences in the abundance level between resistant and susceptible genotypes. Out of 12, nine metabolites were significantly up-regulated in the resistant genotype compared to the susceptible genotype. For all the up-regulated metabolites except Erythrodiol, their role in plant-pathogen interaction was identified as either antimicrobial ((ethylene glycol, chlorogenic acid, trifolin), antiviral activity (diphenyl sulfone, 2-amino oxazole), antifeedant (betulin), changes in the specific biochemical and structural property (xylose) or involvement in signaling cascade (oleic acid). These metabolites act as a metabolic biomarker; their interaction with specific molecular targets associated with MYMV infection can be further examined and utilized to rapidly develop MYMV -resistant cultivars in mungbean.

## Keywords

biomarkers; GC- MS; metabolomics; mungbean; MYMV resistance

## Introduction

Mungbean [*Vigna radiata* (L.) Wilczek], often called 'poor man's meat', has gained significance as a major nutritional supplement besides having a good ability to survive in harsh production environments and nutrient-poor soils (1, 2). This legume is a rich source of proteins (24 %), dietary fiber (64%), calcium (13%), magnesium (47%), vitamins and minerals (3).

However, mungbean cultivation encounters a significant production challenge caused by yellow mosaic disease (YMD) (4). Although YMD incidence has been documented globally, its prevalence is notably higher in countries

such as India, Bangladesh and Pakistan. In India, YMD is primarily caused by two distinct virus species: Mungbean yellow mosaic virus (MYMV) and Mungbean yellow mosaic India Virus (MYMIV) (5). The prevalence of MYMIV disease is notably higher in northern, central and eastern regions of India, while other areas in the peninsular region predominantly exhibit MYMV disease. The primary emphasis of the present research is on MYMV because of its prevalence in Southern India (Tamil Nadu). The mode of disease transmission occurs primarily through whiteflies (*Bemisia tabaci*). Environmental factors, including temperature, humidity and light intensity, impact vector populations and their capacity to transmit disease. The extent of yield loss depends upon the time of infection and the severity of the disease, with severe cases resulting in a notable reduction ranging from 85 to 100% (6). The prime strategy for controlling MYMV disease involves limiting the vector population using agrochemicals. The spray time, particularly during the early vegetative growth, is crucial for controlling vectors. But, spraying at the vegetative stage is often overlooked in farmers' fields, resulting in severe disease incidence. In addition, the persistent use of pesticides can lead to the resurgence of insect vectors and negatively impact the environment and human health. Alternatively, breeders should focus on breeding for resistant varieties that offers a pre-emptive, reliable and sustainable solution. Numerous research efforts have focused on transferring resistant genes through conventional breeding strategies up to the present day. However, studies pertaining to the metabolomic approach, specifically focusing on metabolites and metabolic pathways that could potentially serve as gateways for screening targeted metabolites for MYMV disease resistance are lacking in mungbean.

In plant-pathogen interactions (Genotypes – MYMV), a sequence of biochemical, physiological, mechanical and molecular events is triggered as a disease response. Subsequently, plants exhibit distinct metabolite profiles in resistant and susceptible genotypes. The GC-MS is a potent analytical tool for uncovering metabolites and phytocompounds responsible for systemic defense and acclimation responses during biotic stress (7). Therefore, the present study aims to compare metabolic profiling between resistant and susceptible cultivars upon MYMV infection to enable the identification of metabolic biomarkers responsible for resistance to MYMV.

## Materials and Methods

### *Plant materials and confirmation screening for MYMV disease reaction*

The two mungbean genotypes, GAM 5 and ADT 3, were used in the present study. Based on the previous studies, GAM 5 was identified as a MYMV disease-resistant genotype released from Anand Agricultural University, Gujarat, India. ADT 3 was recognized as a MYMV disease susceptible genotype released from Tamil Nadu Rice Research Institute, Aduthurai, Tamil Nadu, India.

In the present study, MYMV disease response of the above two genotypes was also confirmed by disease screening at two locations, *viz.,* Department of Pulses. Tamil Nadu Agricultural University, Coimbatore (11°N and 77°E) and National Pulses Research Centre, Vamban (10°N and 78°E) during summer, 2023 (D/S: 21.03.2023) and kharif, 2023 (D/S: 15.07.2023), respectively. The infector row technique was utilized for screening. No pesticides were sprayed to maintain a healthy natural whitefly (vector) population, although all other recommended cultivation practices were adhered to. When MYMV disease incidence was recorded in 90 percent of the infector row, disease reaction screening was done in the test genotypes. The genotypes were grouped into resistant and susceptible categories based on Mayee and Datar (8).

### *Metabolome analysis*

Experimental materials: The leaf samples of resistant (no MYMV disease symptoms) and susceptible (MYMV disease symptoms) GAM 5 and ADT3 genotypes were collected from the field at Vamban and rapidly frozen in liquid nitrogen and subsequently stored at -70°C. The frozen powdered samples were then utilized for metabolomic profiling through GC-MS analysis. The analysis was conducted in the Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Sample preparation and metabolite extraction: It includes three steps, *viz.,* extraction, fractionation, and derivatization (9). During extraction, the powdered leaf samples weighing 300 mg were transferred to 2 mL microcentrifuge tubes, followed by the addition of 1.4 mL of 100% methanol. Further, the vortexed mixture was suspended in 50 µL of internal polar standard, ribitol (0.2 mg/mL of water) and incubated at 70°C for 15 min with continuous shaking. This was followed by centrifugation at 12000 rpm for 20 minutes. The supernatant was filtered through a 0.2 µm filter to which 750 µL of chloroform and 1.4 mL of water were added. After thoroughly mixing, the contents were centrifuged at 12000 rpm for 10 minutes to separate the polar and non-polar metabolites. It was followed by fractionation. The upper polar phase (1 mL) was collected in 1.5 mL microcentrifuge tubes. The aliquot was then concentrated (vacuum dried) using a vacuum concentrator at 45°C for 3 hours. The desiccated polar phase was added with 50 µL of methoxyamine hydrochloride (20 mg/mL of pyridine) and incubated at 30°C for 2 hours with continuous shaking for derivatization. Then, N-methyl-N-(trimethylsilyl) trifluoroacetamide (80 µL - MSFTA) was added and incubated at 37°C for 30 minutes and centrifuged at 1,000 rpm for 3 minutes. The supernatant collected was then transferred to GC-MS vials for analysis.

GC-MS analysis: One microliter (1 µL) of the metabolite extracted (sample) was injected into the “PERKIN ELMER CLARUS SQ8C” instrument, equipped with a DB-5 MS Capillary standard non-polar column for the separation of metabolites. The carrier gas used was helium. Initially, the metabolite extracted was vaporized and then separated during analysis. Each compound ideally generated a unique spectral peak, which was digitally documented. Retention time, representing the duration between elution and injection, served as a distinguishing factor for the

compounds. The peak value was computed by assessing the measurement from the peak's base to its apex. The data system incorporates embedded libraries for spectrum detection and matching. NIST MS Search 2.2v encompasses nearly 500,000 references. The interpretation of the mass spectrum from the GC-MS utilized the National Institute of Standards and Technology database (NIST14).

Data analysis: A data table matrix with all the metabolite detected along with retention time and area was obtained as an output. The spectral peak obtained from two mungbean samples (GAM 5 and ADT 3) was compared with known spectra from the NIST and PubChem Databases. Key attributes of the metabolites detected in the sample, including molecular weight, molecular formula and molecular structures, were identified. In addition, the class and sub-class of the metabolites were documented. A Venn diagram was constructed to visualize the number of unique and common metabolites profiled between the resistant and susceptible cultivars using the web tool, "Venny version 2.1" (10). Further, the common metabolites profiled were subjected to PCA analysis using R software version 4.3.3. Fold change analysis was performed to quantify the variation in the abundance level of metabolites between resistant and susceptible genotypes. Data pre-processing and subsequent fold change analysis were performed utilizing the web platform "MetaboAnalyst version 4.0" (11). The significantly up-regulated and down-regulated metabolites between the resistant and susceptible genotypes were determined. Metabolites exhibiting $Log_2$ (FC) > 1 were classified as up-regulated, while those with $Log_2$(FC) < 1 were categorized as down-regulated.

## Results

MYMV disease scoring indicated that the GAM 5 was resistant throughout the crop growth period, displaying no observable disease symptoms in summer, 2023 (Coimbatore) and kharif, 2023 (Vamban) (Table 1). However, the percent disease incidence of ADT 3 was 100% irrespective of the seasons and locations (Table 1). Hence, there was no significant change in the disease reaction category of the two genotypes across two seasons in different locations. Therefore, these two extreme classes of genotypes (resistant and susceptible) were utilized for metabolomic analysis to understand the role of biomolecules in host-plant resistance with respect to MYMV infection in mungbean.

**Table 1.** MYMV disease screening of two mungbean genotypes in two locations

| S. No. | Genotypes | Percent disease incidence (%) | | Category |
|---|---|---|---|---|
| | | Summer, 2023 (Coimbatore) | Kharif, 2023 (Vamban) | |
| 1 | GAM 5 | 0.00 (1) | 0.00 (1) | Resistant |
| 2 | ADT 3 | 100.00 (9) | 100.00 (9) | Highly susceptible |

(Values within parenthesis indicate MYMV scoring scale)

### *Metabolome analysis*

A total of 40 and 49 metabolites were profiled in GAM 5 and ADT 3, respectively (Fig. 1 & 2). These metabolites in GAM 5 and ADT 3 were categorized under 14 and 18 sub-classes, respectively (Fig. 3 & 4). The highest number of metabolites in both genotypes were classified under carbohydrates and their conjugates.

A Venn diagram showed that 27 metabolites profiled by GC-MS were shared by GAM 5 and ADT 3 (Fig. 5 and Supplementary Table 1). In addition, 13 compounds were uniquely profiled in GAM 5 (Fig. 5), and 22 compounds were unique to the susceptible variety, ADT 3 (Fig. 5). The 27 common metabolites profiled in GAM 5 and ADT 3 were subjected to PCA analysis and an apparent separation was observed between resistant and susceptible genotypes. The result of PCA analysis is depicted in Fig. 6. It was also observed that the metabolites *viz.,* ethylene glycol, chlorogenic acid, oleic acid and trifolin were specific to GAM 5 (Fig. 6).

However, the significant changes in the abundance level of 27 metabolites profiled in GAM 5 and ADT 3 were further identified through fold change analysis. Among these 27 metabolites, 12 compounds exhibited a significant difference in the peak area (abundance level) (Table 2). Specifically in the resistant genotype GAM 5, nine compounds *viz.,* ethylene glycol, chlorogenic acid, diphenyl sulfone, 2-amino oxazole, betulin, erythrodiol, xylose, oleic acid and trifolin were significantly up-regulated with $Log_2$

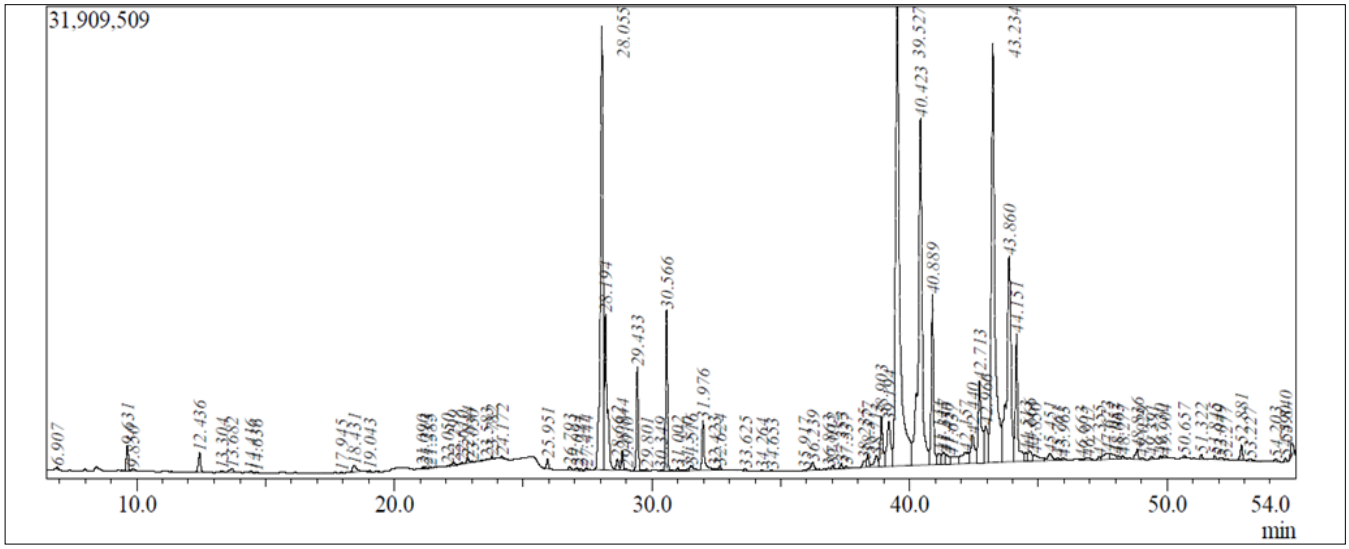


**Fig. 1.** Chromatogram of methanol extract of MYMV infected leaves of GAM 5 (resistant).

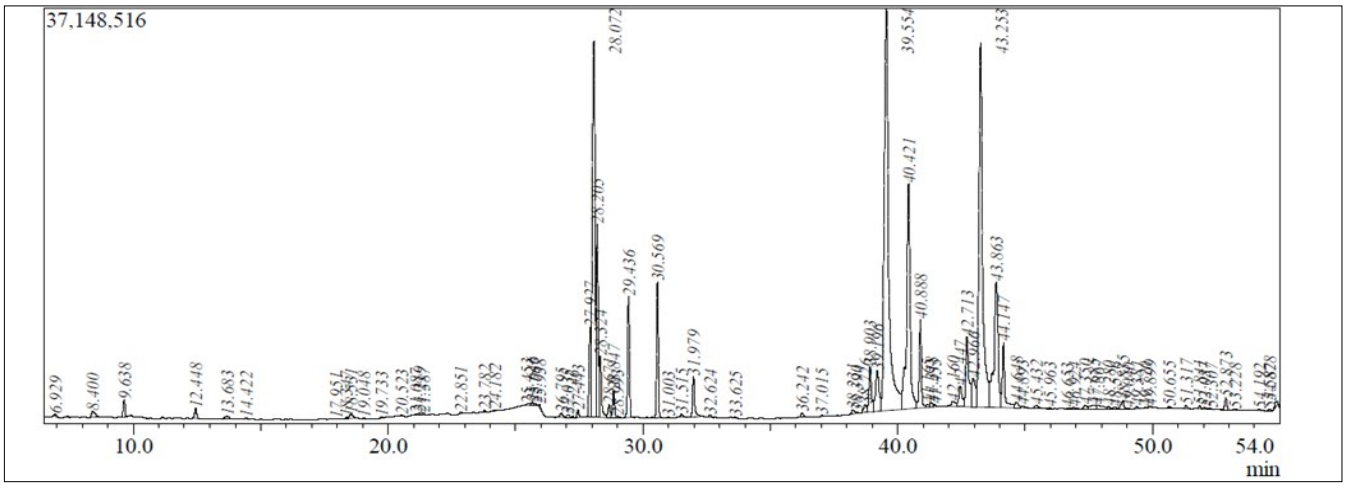


**Fig. 2.** Chromatogram of methanol extract of MYMV infected leaves of ADT 3 (susceptible).

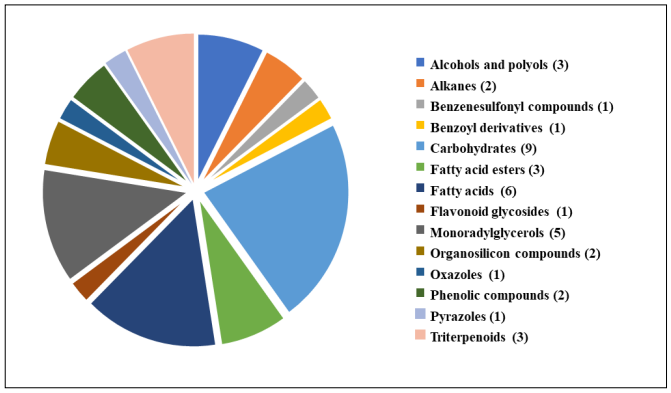


**Fig. 3.** Classification of metabolites profiled in resistant genotype (GAM 5).

(Values within parenthesis represent number of metabolites profiled under each group)

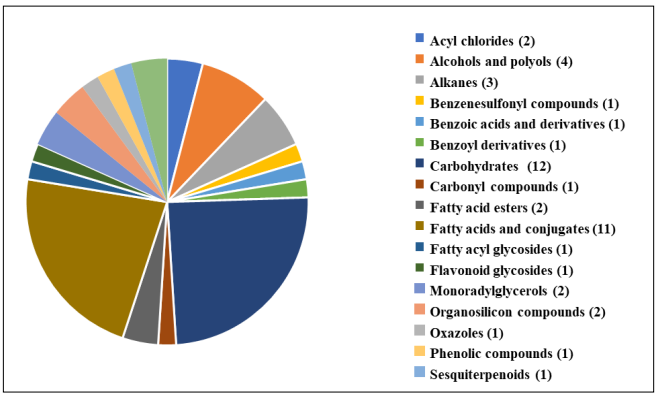


**Fig. 4.** Classification of metabolites profiled in susceptible genotype (ADT 3).

(Values within parenthesis represent number of metabolites profiled under each group)

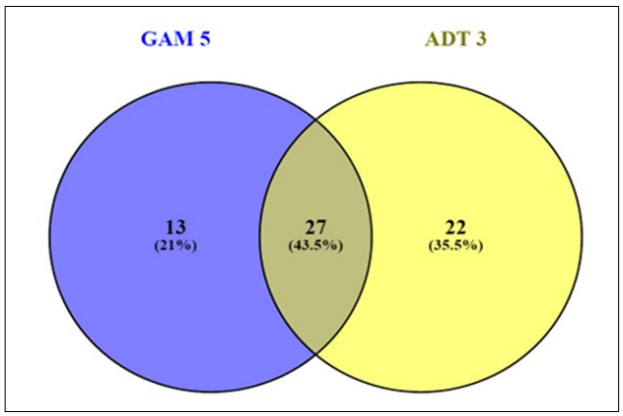


**Fig. 5.** Venn diagram representing number of metabolites accumulated [GAM 5 (R) vs ADT 3 (S)].

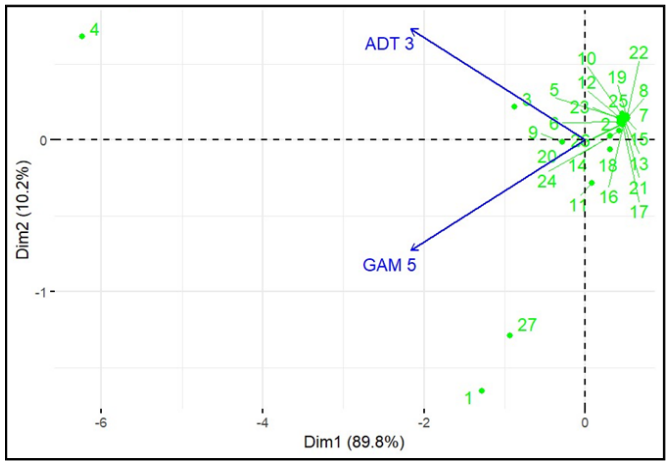


**Fig. 6.** Principal component analysis of metabolites.

**Table 2.** Fold change analysis of common metabolites profiled in MYMV-resistant genotype (GAM 5)

| S. No. | Compound name | Compound Id | Fold Change | $Log_2$ (FC) |
|---|---|---|---|---|
| **Uprgulated** | | | | |
| 1. | Ethylene glycol | 174 | 245.970 | 7.942 |
| 2. | Chlorogenic acid | 1794427 | 73.996 | 6.209 |
| 3. | Diphenyl sulfone | 31386 | 35.656 | 5.156 |
| 4. | 2-Aminooxazole | 281137 | 28.910 | 4.854 |
| 5. | Betulin | 72326 | 6.425 | 2.684 |
| 6. | Erythrodiol | 101761 | 5.335 | 2.415 |
| 7. | Xylose | 135191 | 4.130 | 2.046 |
| 8. | Oleic acid | 445639 | 3.012 | 1.591 |
| 9. | Trifolin | 5282149 | 2.318 | 1.213 |
| **Down-regulated** | | | | |
| 1. | Arachidonic acid | 444899 | 0.077 | -3.709 |
| 2. | Methyl arachidonate | 6421258 | 0.238 | -2.075 |
| 3. | 2,3-Dimethyl-para-anisaldehyde | 596058 | 0.413 | -1.276 |

**Table 3.** Details of significantly up-regulated and down-regulated metabolites in GAM 5 (R) in comparison to ADT 3(S) genotype

| S. No. | Compound name | PubChem ID | Chemical group | Molecular formula | Molecular weight (g/mol) | Peak area (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | GAM 5 (R) | ADT 3 (S) |
| **Uprgulated** | | | | | | | |
| 1. | Ethylene glycol | 174 | Alcohols and polyols | $C_2H_6O_2$ | 62.07 | 10.72 | 0.03 |
| 2. | Chlorogenic acid | 1794427 | Alcohols and polyols | $C_{16}H_{18}O_9$ | 354.31 | 8.60 | 0.08 |
| 3. | Diphenyl sulfone | 31386 | Benzene sulfonyl compounds | $C_{12}H_{10}O_2S$ | 218.27 | 2.59 | 0.05 |
| 4. | 2-Aminooxazole | 281137 | Oxazoles | $C_5H_5N_3O$ | 123.11 | 1.26 | 0.03 |
| 5. | Betulin | 72326 | Triterpenoids | $C_{30}H_{50}O_2$ | 442.70 | 0.56 | 0.06 |
| 6. | Erythrodiol | 101761 | Triterpenoids | $C_{30}H_{50}O_2$ | 442.70 | 0.31 | 0.04 |
| 7. | Xylose | 135191 | Carbohydrates | $C_5H_{10}O_5$ | 150.13 | 0.30 | 0.05 |
| 8. | Oleic acid | 445639 | Fatty acids | $C_{18}H_{34}O_2$ | 282.50 | 0.35 | 0.08 |
| 9. | Trifolin | 5282149 | Flavonoid glycosides | $C_{21}H_{20}O_{11}$ | 448.40 | 1.01 | 0.30 |
| **Down-regulated** | | | | | | | |
| 1. | Arachidonic acid | 444899 | Fatty acids | $C_{20}H_{32}O_2$ | 304.50 | 0.01 | 0.09 |
| 2. | Methyl arachidonate | 6421258 | Fatty acid esters | $C_{21}H_{34}O_2$ | 318.50 | 0.10 | 0.29 |
| 3. | 2,3-Dimethyl-para-anisaldehyde | 596058 | Benzoyl derivatives | $C_{10}H_{12}O_2$ | 164.20 | 0.03 | 0.05 |

(FC) > 1 (Table 2 & 3 and Fig. 7). Diagrammatic representation of the up-regulated metabolites is represented in Fig. 7. The remaining three compounds *viz.,* arachidonic acid, methyl arachidonate and 2,3-dimethyl-para-anisaldehyde were significantly down-regulated in the resistant genotype GAM 5 with $Log_2$ (FC) < 1 (Table 2 & 3 and Fig. 8). The graphical view of the three down-regulated metabolites in the resistant genotype is represented in the Fig. 8.

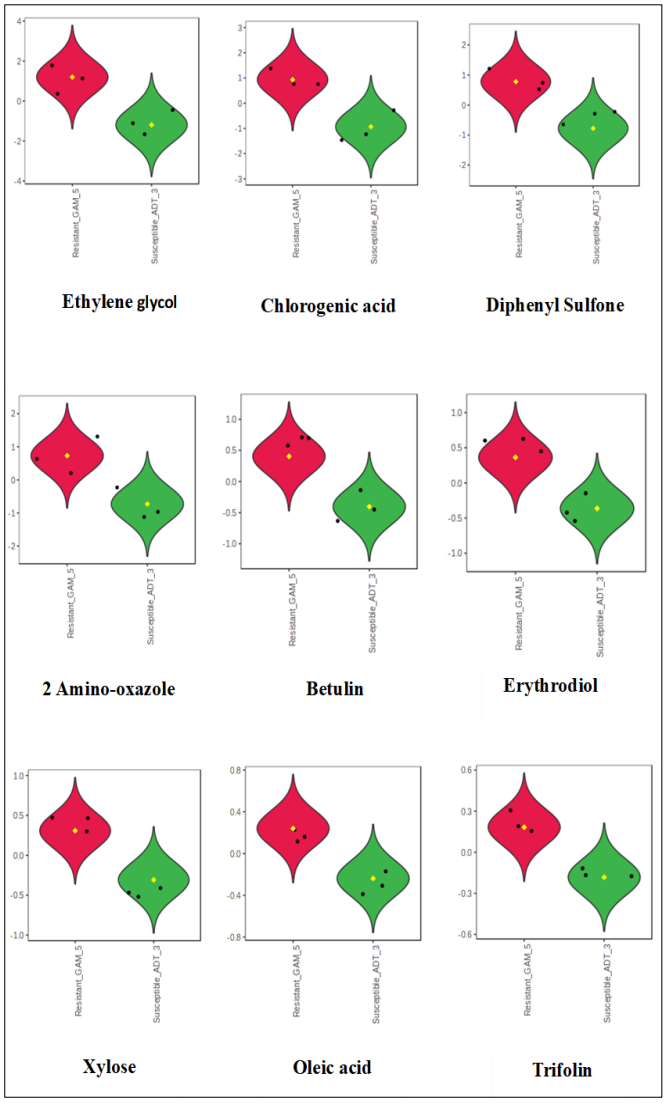


**Fig. 7.** Fold change of nine up-regulated metabolites in GAM 5 (resistant) compared with ADT 3 (susceptible).

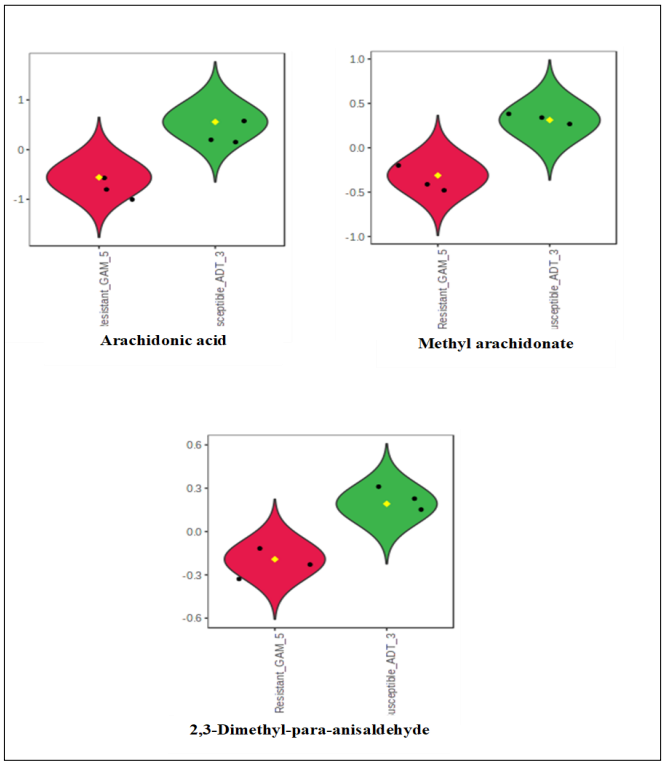


**Fig. 8.** Fold change of three down-regulated metabolites in GAM 5 (resistant) on comparison with ADT 3 (susceptible).

## Discussion

In response to pathogen attacks, plants employ diverse strategies to activate their defense mechanism. Consequently, there are significant changes in both primary and secondary metabolites. In the present study, the selected MYMV-resistant and susceptible genotypes were reconfirmed for their MYMV disease reaction in two locations and used for metabolome analysis. Metabolites belonging to various sub-classes in GAM 5 and ADT 3, likely contribute to different aspects of the plant's defense, including signaling, secondary metabolite production and overall stress response. Notably, both genotypes exhibited the highest number of metabolites in the category of carbohydrates and their conjugates. The alterations in the levels of carbohydrates in response to the pathogen could indicate shifts in energy allocation and utilization as part of the defense mechanisms (12).

Comparative analysis of the metabolite profiled between resistant genotype (GAM 5) and susceptible genotype (ADT 3) revealed specific metabolites that were differentially regulated in the resistant genotype. GAM 5 and ADT 3 shared a common set of 27 metabolites identified by GC-MS analysis. In the fold change analysis of 27 common metabolites, 12 compounds exhibited significant differences in peak area, indicating altered abundance levels. In the resistant genotype GAM 5, nine compounds *viz.,* ethylene glycol, chlorogenic acid, diphenyl sulfone, 2-amino oxazole, betulin, erythrodiol, xylose, oleic acid and trifolin were up-regulated. The up-regulated metabolites in the resistant genotype, GAM 5, may serve as potential biomarkers for resistance and are further discussed for their role in plant-pathogen interactions.

The antimicrobial activity of ethylene glycol was documented in a previous study (13). Accumulation of ethylene glycol was also reported in another study on the YMV-resistant horsegram genotype (PLS6002) (14). The resistant genotype, GAM 5, exhibited elevated levels of chlorogenic acid and showed antimicrobial and antioxidant activity against MYMV. It also acts as a potential botanical insecticide, thereby preventing the spread of MYMV through whitefly transmission (10). Antimicrobial activity of chlorogenic acid against *Mythimna separata*, *Pseudomonas syringae* and *Alternaria alternata* were also documented in tomatoes (15-17). The protective role of chlorogenic acid in plants as antioxidants under adverse conditions by scavenging reactive oxygen species (ROS) was reported in a separate investigation (18).

The up-regulation of diphenyl sulfone and 2-Amino oxazole metabolitesin GAM 5 exhibited significant antiviral property against MYMV. Similarly, in horse gram, the antiviral activity of the above two metabolites against horse gram yellow mosaic virus has also been reported (14).

The relative concentration of triterpenoids (Betulin and Erythrodiol) was higher in GAM 5 than ADT 3 upon MYMV infection. Betulin has been documented for its antimicrobial activity against various pathogens (14). Its active role in resistance against the green-peach aphid has been reported in a study (19). Betulin was also identified as an antiviral terpenoid specifically accumulated in resistant black gram (*Vigna mungo*) cultivars in response to MYMV infection (20). Additionally, betulin has demonstrated an antifeedant effect against bollworm larvae (*Heliothis zea*), Colorado potato beetle and *Callosobruchus chinensis* (21). These findings collectively suggest that betulin may play a crucial role in the resistance mechanism of GAM 5 against MYMV infection. The role of Erythrodiol in plant-pathogen interaction is less understood and requires further research.

Hemicellulose is a group of cell wall polysaccharides composed mainly of xylose, glucose, mannose and galactose. Pectin and structures associated with hemicellulose in plant cell walls actively contribute to defense responses against pathogens (22). The review of the relationship between plant cell wall components, including hemicellulose and plant immunity highlighted that hemicellulose modifications can trigger immune responses and enhance pathogen resistance (23). Therefore, it is inferred that the upregulation of xylose in hemicellulose is posited to confer specific structural and biochemical properties that augment the overall defense mechanism of GAM 5, potentially influencing the plant's resistance against MYMV infection.

Elevated levels of oleic acid, a monounsaturated fatty acid in GAM 5, are a primary source of reserve energy and contribute to the formation of complex lipids, essential components of cellular membranes. Evidence suggests that fatty acids and their derivatives are signaling molecules influencing plants' normal and disease-related physiological processes (24). The role of Oleic acid in defense signaling was reported in *Arabidopsis* (24) and soybean (25) by regulating salicylic acid levels and defense gene expression.

Trifolin, a flavonoid commonly found in various plants, displays both bactericidal and antifungal properties. Additionally, its antiviral efficacy against the cassava mosaic virus has been documented (26). The antimicrobial activity of trifolin against various pathogens *in vitro* was also demonstrated (27).

Collectively, this study identified that higher accumulation of ethylene glycol, chlorogenic acid, diphenyl sulfone, 2-Amino oxazole, betulin, erythrodiol, xylose, oleic acid and trifolin helped GAM 5 to resist the pathogenicity of MYMV and thus emerged as a highly resistant genotype. These metabolites were identified for their role in plant-pathogen interaction by exhibiting antimicrobial activity, changes in specific biochemical and structural properties or involvement in signaling cascade during MYMV infection.

## Conclusion

The comparative metabolic profiling of MYMV disease-resistant and susceptible genotypes resulted in the identification of metabolites *viz.,* ethylene glycol, chlorogenic acid, diphenyl sulfone, 2-amino oxazole, betulin, erythrodiol, xylose, oleic acid and trifolin that confers resistance to MYMV. These metabolites can further be validated for their use as metabolic biomarkers. Thus, identifying metabolic biomarkers and integrating metabolomic tools holds significant scientific value in mungbean's rapidly developing MYMV-resistant cultivars.

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## Authors' contributions

TN has conducted research, including data collection, statistical analysis and interpretation, and has prepared the manuscript. CB has made a substantial contribution to drafting the research program. PJ has been interpreting data and revising the manuscript critically for scientific content. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interest to declare.

**Ethical issues:** None

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