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RESEARCH ARTICLE

Investigation of phenolic and polyphenolic responses in phloem sap of citrus cultivars infected with *Candidatus Liberibacter asiaticus*

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A B S T R A C T

Huanglongbing (HLB), caused by *Candidatus liberibacter asiaticus*, is a destructive citrus disease that disrupts plant physiology and is transmitted by *Diaphorina citri*. It is hypothesized that higher degree phenolic contents showed higher tolerance to many plant pathogens. In this regard, present study was designed to aimed to characterize phenolic and polyphenolic content in the phloem sap of healthy and HLB-infected citrus varieties, including *Citrus reticulata* (Kinnow, Daisy and Nagpur), *Citrus sinensis* (Saddis and Valencia Late), and *Citrus limon* (Lezben). Infection was confirmed through RT-PCR, and phenolic content was analyzed using Gas Chromatography–Mass Spectrometry (GC–MS). A total of 79–137 distinct compounds were identified per cultivar, revealing striking differences in metabolite accumulation. Several phenolics, such as hydroxyhydrocodone, cyclotrisiloxane derivatives, and 2-ferrocenyl-3-(m-tolyl)thiazolidin-4-one, were exclusively abundant in healthy peels but depleted in infected tissues, suggesting their protective roles in defense mechanism. Conversely, stress-associated metabolites, including oxycodone, 2-methoxy-4-vinylphenol, indole derivatives, and sesquiterpenes like longifolene and aromandendrene, were predominantly induced in *C*Las-infected samples, reflecting pathogen-triggered defense activation. Some compounds (4-Pyridinol, 2,6-bis[p-chlorophenyl], Oxycodone, 2-Methoxy-4-vinylphenol, 3-Methoxyacetophenone, Acethydrazide derivatives) displayed significant fold-changes between healthy and diseased states, with p-values <0.05, underscoring their potential as metabolic biomarkers for early HLB detection. Importantly, cultivars with higher concentration and diversity of phenolic compounds, such as in Kinnow and Valencia Late, demonstrated broader chemical defense responses, supporting the hypothesis that elevated phenolic abundance enhances tolerance against pathogen stress. By employing a multi-varietal comparative approach, this study provides novel evidence of infection-induced metabolic markers that may facilitate the development of early diagnostic tools resistance breeding strategies. Deciphering the mechanisms underlying citrus tolerance to HLB will aid in the development of commercially viable tolerant cultivars.

Keywords: Huanglongbing, Polyphenols, *Candidatus* Liberibacter asiaticus, Phenolics.

INTRODUCTION

Citrus, a diverse genus within the Rutaceae family, is cultivated worldwide for its high-value fruits, which are rich in vitamins, flavonoids, and antioxidants (Ahmad *et al.,* 2024; Hassan *et al.,* 2025). Beyond their nutritional contributions, citrus fruits hold immense economic and industrial importance, serving as raw material for food, nutraceutical, pharmaceutical, and cosmetic industries (Anwar *et al.,* 2021; Palangasinghe *et al.,* 2024). Regular citrus consumption has been associated with reduced risk of cardiovascular disorders, metabolic syndromes, and

several cancers, underscoring its value to human health (Bhatt *et al.,* 2024). However, global citrus production is increasingly threatened by biotic and abiotic stresses, among which Huanglongbing (HLB), or citrus greening disease, is the most devastating (Limayem *et al.,* 2024; Djeddour *et al.,* 2021).

Hunaglongbing (HLB) is caused by the fastidious, phloem-limited bacterium *Candidatus liberibacter asiaticus* (*C*Las), transmitted by the Asian citrus psyllid (*Diaphorina citri*). Since its rapid global spread, HLB has caused severe yield declines, fruit deformities, premature fruit drop, and tree mortality, making it the greatest challenge to sustainable citrus production (Hosseinzadeh & Heck, 2023; Li & Nangong, 2022: Sultan *et al.,* 2025). At the physiological level, *C*Las disrupts phloem function, impairs nutrient allocation, and induces chronic metabolic stress, ultimately leading to canopy loss and economic decline (Huang *et al.,* 2025; Welker *et al.,* 2022). Despite intensive efforts, there is currently no sustainable cure for HLB, and management remains largely limited to vector control and cultural practices, both of which are insufficient (Alquézar *et al.,* 2022; Pérez-Hedo *et al.,* 2025).

Recent advances in omics technologies have highlighted CLas responses to host metabolism, particularly pathways linked to carbohydrates, amino acids, and defense-related metabolites. Among these, phenolics and polyphenolics are of particular interest due to their roles in antioxidation, lignification, antimicrobial defense, and systemic acquired resistance (SAR). Evidence suggested that *C*Las infection alters phenolic biosynthesis, either by suppressing protective compounds or inducing novel stress-related metabolites (Serag *et al.,* 2022; War *et al.,* 2024; Herranz *et al.,* 2024). However, most studies have focused on leaves or whole tissues, while comparative analysis of phenolic and polyphenolic composition specifically in phloem sap across multiple citrus cultivars remains limited. Since the phloem is the primary site of pathogen colonization, understanding these biochemical shifts is critical to unraveling host–pathogen interactions (Dandlen *et al.,* 2023; Gaikwad *et al.,* 2025; Gross *et al.,* 2022). Despite growing recognition of phenolic compounds as potential defense biomarkers, their variability across healthy and infected cultivars has not been systematically characterized. This knowledge gap limits our ability to identify reliable metabolic markers for early HLB detection or to harness natural biochemical resistance in breeding programs. The present study addresses this gap by profiling and comparing phenolic and polyphenolic metabolites in the phloem sap of healthy and HLB-infected citrus cultivars. By correlating infection severity with metabolites variation. This study aims to identify key biomarkers associated with host defense and cultivar susceptibility. Ultimately, this work provides a foundation for developing biochemical diagnostic tools and informs breeding strategies for HLB-affected citrus varieties, offering long-term solutions for sustainable citrus production.

MATERIALS AND METHODS

The experiments in this study were designed in a stepwise manner to establish a clear link between *Candidatus liberibacter asiaticus* (*C*Las) infection and the phenolic/polyphenolic profiles of citrus phloem sap. First, infection status was confirmed by RT-PCR to ensure accurate classification of healthy and HLB-infected samples, since symptom-based diagnosis alone can be misleading. Phloem sap was then extracted, as it is the primary site of *C*Las colonization and the most relevant tissue for studying host–pathogen interactions. Quantification of total phenolics and polyphenolics was performed to assess overall defense-related metabolite accumulation, while advanced profiling using GC-MS enabled the identification of specific compounds that may act as biomarkers of resistance or susceptibility. Comparative analysis between healthy and *C*Las-infected cultivars provided insights into genotype-specific responses and the extent to which metabolite composition influences tolerance to HLB. Finally, statistical and correlation analyses were conducted to validate the significance of observed biochemical differences and to establish associations between infection severity and metabolite levels. Collectively, these experiments were performed to uncover the biochemical basis of citrus defense, identify key metabolites linked to disease resistance, and provide a foundation for developing metabolite-based management or breeding strategies against HLB.

Collection of plant materials and extraction of phloem sap: For this study, the three citrus species were collected from the, Citrus Research Institute (CRI), Sargodha, Bhalwal, namely, *Citrus sinensis* varieties (Saddis and Valencia Late), *Citrus limon* verities (Lezben), and *Citrus reticulate* varieties (Nagpur, Kinnow and Daisy). The stems of between 10 and 20 cm long and 0.2 and 0.3 cm in diameter were used for the extraction of phloem sap. The protocol for the phloem extraction was followed by

Killiny (2019).

Identification of infection status through RT-PCR: The determination of the *C*Las-infection status was conducted by qPCR utilizing SYBR Green (Li *et al.,* 2020) (Appendix I). The primer set LJ900 (Forward: 5′-CAGTCGAGCGTTAACGTTG-3′, Reverse: 5′-ACGGTAGAGCTTCCGTCTT-3′) produced an amplicon derived from the repeated copies of the prophage present within the *Candidatus* Liberibacter asiaticus (*C*Las) genome. Forward and reverse primer concentrations were 0.6 µM and 1 µM, respectively, and the annealing temperature was 62°C. Unless otherwise indicated, 2 µL of each DNA preparation was used per 25 µL reaction. SYBR Green-based (Qunta Biosience, Inc.). Real-time PCR was run at 95°C for 10 min, followed by 40 cycles of 95°C for 30sec, 62°C for 1min, 68°C for 1 min and terminated by 95°C for 1min, 62 °C for 30s and 68°C for 30sec, with fluorescence signal capture at the end of each 62°C step. The specificity of the reactions was monitored by analysis of the thermal melt profiles. All PCR reactions were conducted using the Agilent Technologies, Inc. M×3005P, Fast real-time PCR system. Cycle threshold (Ct) values were determined using ABI 7500 Software version 2.0.1 with a manually set threshold at 0.1 and automated baseline settings (Bao *et al.,* 2020).

Identification of phenolic and polyphenolic compounds through GC-MS: The polyphenols were quantified using the GC-MS, protocols. For the analysis, aliquots of the fluids from 05 samples of each variety were dried and the remaining dry weight was redisposed in pyridine mixture of methoxyamine hydrochloride. Then, 70 µL of MSTFA (N-Methyl-N [trimethylsilyl]trifluoroacetamide) and 6 µL of a standard retention time mixture (a 3.7% [w/v] solution of fatty acid methyl esters with several carbons from 8 to 24) were added. The samples were further placed in the incubator for 30min, except for the control sample which was incubated for only 15 minutes. The prepared sample was injected into an Agilent Technologies 6890 N gas chromatograph in split 1:30 and split less modes; connected to LECO Pegasus 4D TOF mass spectrometer. The flow rate was 2 mL/min and the samples were injected to the BCX35 column with the following dimensions: length 30 m, inner diameter 0. 32 mm, film thickness 0. 25 µm manufactured by SGE Analytical Science Pty Ltd, Australia and the gas used was Helium (He). As for the inlet temperature it was set at 250°C. The oven temperature time profile included a gradient ramp of 8°C/min to 360 °C and gradient 85 °C for 2 min. Ionization was performed at 70 eV, and the range of the mass spectra was varied from 35– 900 Da, with a scanning rate of 6. 25 spectra per second. Data acquisition and data analysis of chromatographic data were done using CHROMATOF 6. 2 software from LECO, Inc (Monteiro *et al.,* 2022).

RESULTS

Citrus samples of different species such as *Citrus sinensis* varieties (Saddis and Valencia), *Citrus limon* verities (Lezben), and *Citrus reticulate* varieties (Nagpur, Kinnow and Daisy). were chosen for this study. The DNA extraction of leaves from different citrus cultivars made it possible to screen a clear distinguished between healthy and *C*Las infection. A clear distinguished between *C*Las-positive and *C*Las-negative samples were observed through different Ct values. Six samples were observed as *C*Las -positive (Ct Values) ranged from 15.5 (Lezben) to 28.2 (Kinnow). Most of the healthy samples have no Ct value or greater than 30 Ct value and were perfectly considered as negative for *C*Las-infection status. The concentration of DNA in citrus cultivars was calculated from spectrophotometers and were observed the highest in *C*Las-negative sample, Lezben, i.e., 292 ng/µl and lowest in Kinnow, i.e., 1.469. Likewise, *C*Las-negative samples ranged from 23 ng/µl (Saddis) to 295 ng/µl (Kinnow). The copy number of *Candidatus* liberibacter asiaticus (*C*Las) DNA (prophage-specific region) was quantified using a standard calibration curve generated from serial dilutions of a gel-purified amplicon. In healthy citrus samples, Ct values ranged from 36.3 to 15.5, corresponding to copy numbers between 0 and 139. Following previously established thresholds (Paula *et al.,* 2018; Sieburth *et al.,* 2009), samples with ≤139 copies or Ct values ≥30 were considered negative, as such levels are below the reliable detection limit. In contrast, infected samples (*C*Las-infected) exhibited markedly higher copy numbers, frequently in the range of millions, with a maximum of 4.5×10^6 copies (Ct = 16.50). Exceptions were observed in three samples, where copy numbers ranged from 1.39×10^3 (Ct = 28.2) to 1.87×10^5 (Ct = 21.1). This figure 1 showed RT-PCR amplification plots representing the increase in fluorescence signal (y-axis) as a function of RT-PCR cycle number (x-axis). Each curve corresponds to a different replicate of citrus cultivars.

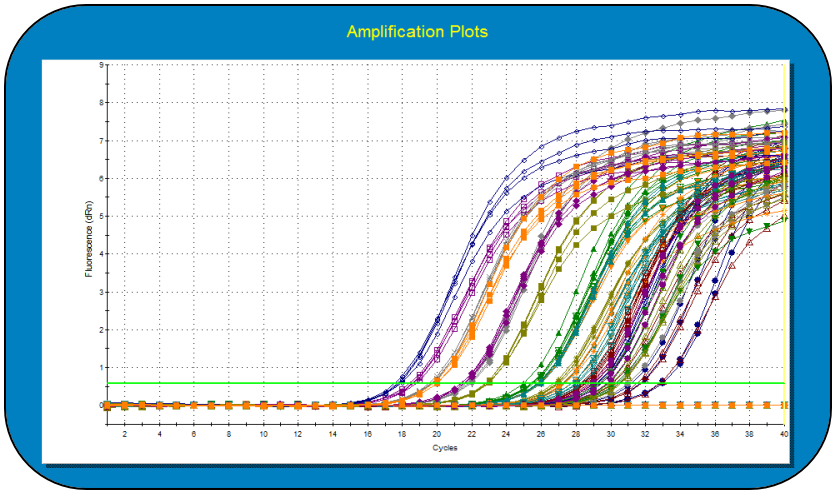

Figure 1 (a). RT- PCR results indicating the Huanglongbing- infection status in citrus cultivars. Legend: b) A representative amplification curve produced in real time by RT-PCR using three wells per sample from *C*Las -infected and healthy citrus cultivars.

The next phase of this study involved the analysis of phenolic compounds in the phloem sap of different citrus cultivars through spectrometry (GC-MS). This analysis aimed to explore the potential role of phenolic compounds in influencing *C*Las infection severity.

***Citrus reticulata* var. Kinnow:** Metabolomic profiling of Kinnow mandarin peel revealed a clear involvement of stress-induced phenolics in *C*Las-infected samples compared to healthy tissues. Compounds such as Tryptamine (55.1 µg/g), 2-(Ethyl)oxybenzylidene acetophenone (207–377 µg/g), Morphinan-4-ol-6,7-dione, 3-methoxy-N-methyl (315 µg/g), and 3-(3,7-Dimethylocta-2,7-dienyl)-1H-indole (up to 377 µg/g) were exclusively detected in infected tissues. Additionally, Oxycodone and 4-Pyridinol, 2,6-bis[p-chlorophenyl]- were significantly upregulated (from 55.1 µg/g in healthy to 315 µg/g in infected). These findings indicate pathogen-triggered induction of defense-related secondary metabolism. In contrast, 6-Aminochrysene and 2-ferrocenyl-3-(m-tolyl)thiazolidin-4-one was strongly depleted under infection stress, supporting the notion of selective suppression of baseline protective metabolites. The figure 2 compared the chromatographic profiles of phenolic metabolites in *Citrus reticulata* var. Kinnow phloem sap, showed distinct peak patterns between healthy and *C*Las-infected samples.

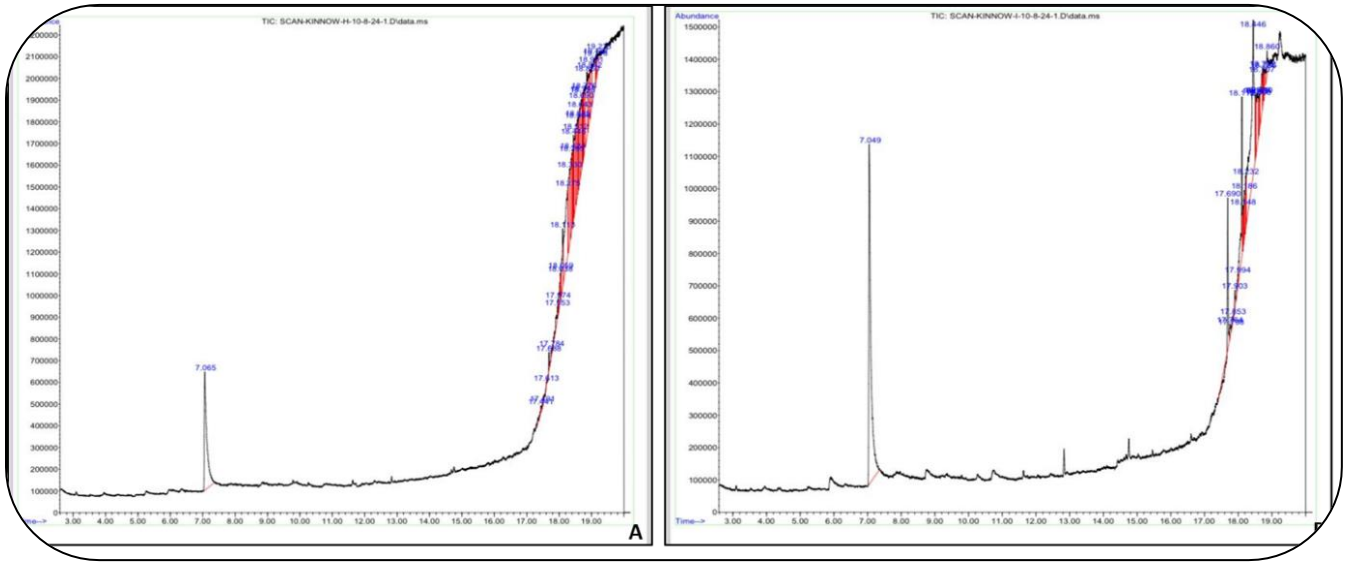

Figure 2. Chromatographic peaks representing phenolic metabolites profile in *Citrus reticulata* var. Kinnow. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

***Citrus reticulata* var. Nagpur:** In Nagpur mandarin, infection was associated with the exclusive accumulation of Oxycodone and 2-[2-[2-(4-Chlorophenoxy)-ethylsulfanyl]-benzoimidazol-1-yl]-acetamide, which were absent in healthy peels. Conversely, key compounds such as Hydroxyhydrocodone and Cyclotrisiloxane derivatives were lost upon infection. The pronounced switch from protective phenolics in healthy tissues to pathogen-induced metabolites in infected tissues reflects a metabolic development under bacterial stress. Figure 3 compared the chromatographic profiles of phenolic metabolites in *Citrus reticulata* var. Nagpur phloem sap, showed different peak configurations between healthy and *C*Las-infected samples.

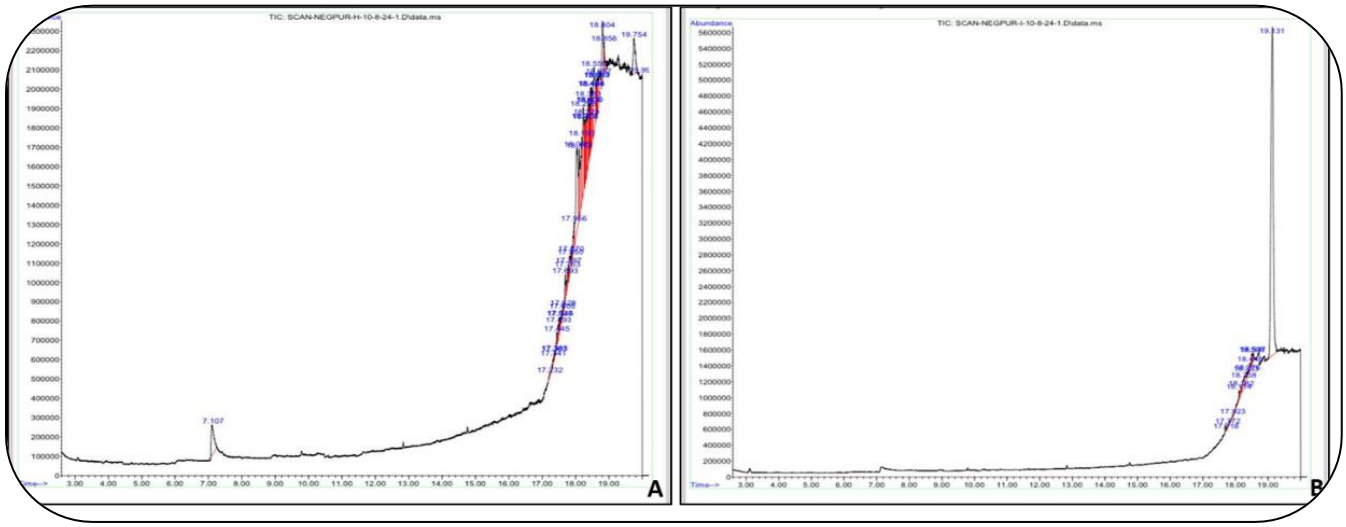

Figure 3. Chromatographic peaks representing phenolic metabolites profile in *Citrus reticulata* var. Nagpur. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

***Citrus reticulata* var. Daisy:** The Daisy cultivar demonstrated a distinct reprogramming of polyphenols under HLB infection. Infection induced significant enrichment of Oxycodone, 2-Methoxy-4-vinylphenol, and 3-Methoxyacetophenone, while compounds such as Valerenol, Cyclotrisiloxane derivatives, and Caryophyllene-(I1) were completely absent in diseased tissues. The appearance of sesquiterpenes including Longifolene, Aromandendrene, and Azulene derivatives exclusively in infected samples suggests a defense-driven metabolic relocation. The figure 3 compared the chromatographic profiles of phenolic metabolites in *Citrus reticulata* var. Daisy phloem sap, showed distinct peak patterns between healthy and *C*Las-infected.

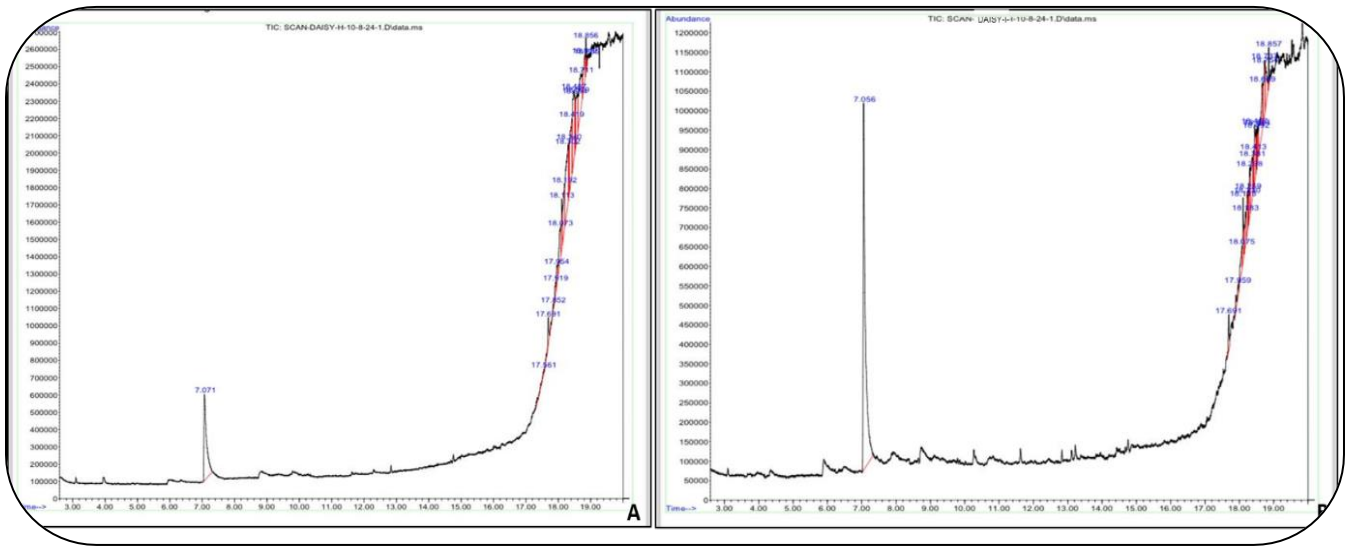

Figure 3. Chromatographic peaks representing phenolic metabolites profile in *Citrus reticulata* var. Daisy. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

***Citrus sinensis* var. Saddis:** Saddis phloem sap showed pronounced metabolic shifts with infection. 2-Methoxy-4-vinylphenol, 3-Methoxyacetophenone, Acethydrazide derivatives, fluorinated phenylacetamides, and 4-epi-Dehydroabietinol acetate were significantly enriched in infected tissues, while protective metabolites including Oxycodone, trans-3,4,5-Trimethoxy-β-methyl-β-nitrostyrene, and cyclotrisiloxane derivatives were strongly depleted. These shifts suggested that HLB affecting phenolic biosynthesis towards novel stress-induced metabolites while suppressing pre-existing defense metabolites. Figure 4 represented the chromatographic peaks of phenolic metabolites in *Citrus sinensis* var. Saddis phloem sap, which showed different peak patterns between healthy and *C*Las-infected.

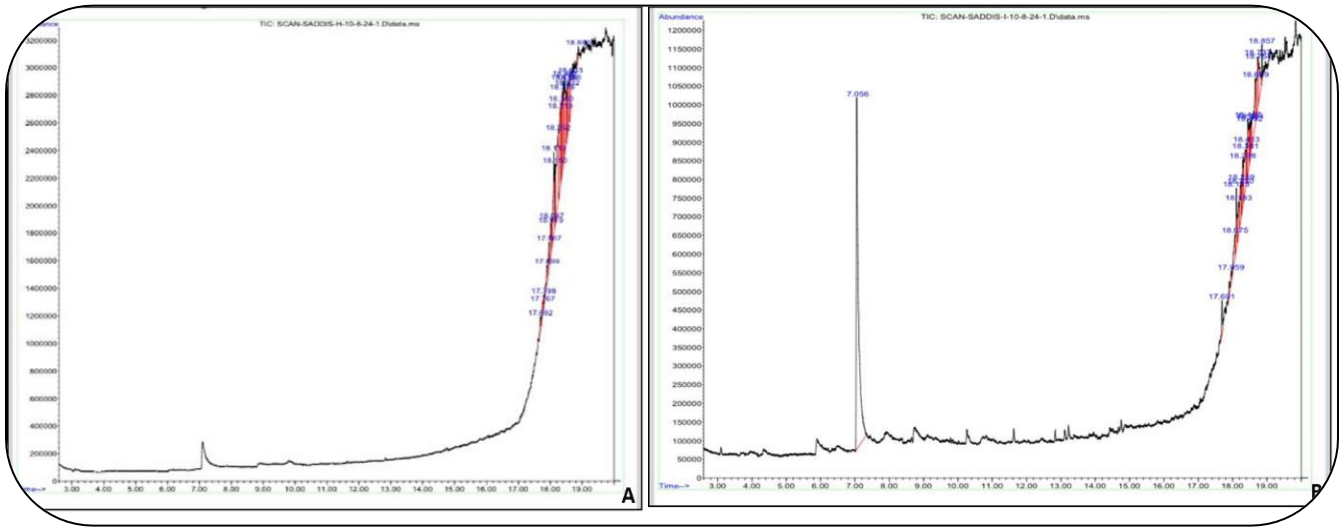

Figure 4. Chromatographic peaks representing phenolic metabolites profile in *Citrus sinensis* var. Saddis. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

***Citrus sinensis* var. Valencia Late:** Valencia Late displayed marked depletion of Trifluoroacetyl-epiisoborneol, Valerena-4,7(11)-diene, and Caryophyllene-(I1) in infected tissues, while infection induced accumulation of Oxycodone, 2-Methoxy-4-vinylphenol, and Naphtho[1,2-b]quinoxaline, 5-(4-morpholyl). This pattern reflects pathogen-driven suppression of sesquiterpenes and parallel induction of alkaloid- and phenolic-rich defense chemistry. The chromatographic peaks of phenolic metabolites in *Citrus sinensis* var. Valencia Late phloem sap were seen in fig 5 which showed different peak patterns between healthy and *C*Las-infected.

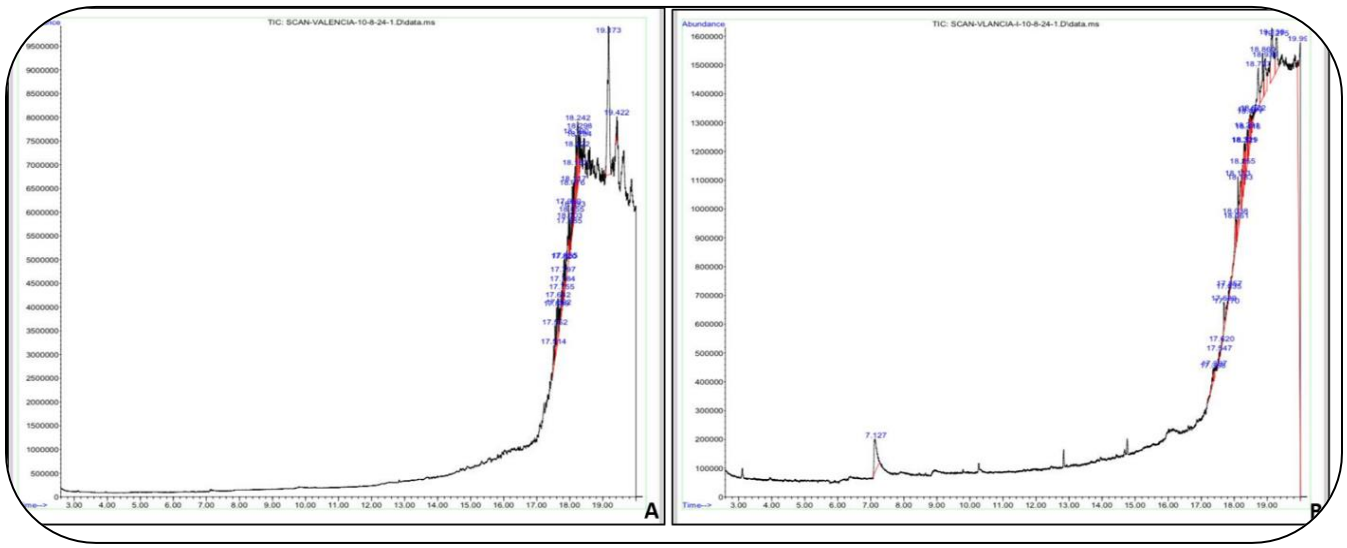

Figure 5. Chromatographic peaks representing phenolic metabolites profile in *Citrus sinensis* var. Valencia Late. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

***Citrus limon* var. Lezben:** In Lezben lemon, multiple phenolic compounds including 6-Methyl-2-(3-nitrophenyl)imidazo[1,2-a]pyridine and Cyclotrisiloxane derivatives were present in healthy tissues but absent in infected samples, while infection induced the primary biosynthesis of Pancracine, N-Methyl-1-adamantaneacetamide, and thiazole derivatives. These metabolites likely represented the stress-activated defense ranges that are absent in the normal bio-physiological state in citrus cultivars. Figure 6 indicated the distinct peak (m/z vs. time) pattern in *citrus sinensis* var. Lezben.

Table 1 indicated the differential accumulation of polyphenolic metabolites in healthy vs. *C*Las-infected in all citrus cultivars collectively. Several metabolites such as tryptamine, oxycodone, and naphthoquinoxaline were induced or upregulated in *C*Las-infected samples; However, other metabolites i.e., hydroxyhydrocodone, caryophyllene, and certain siloxane derivatives were not detected upon the severity of infection. Statistical analyses (t-test and One-Way ANOVA) confirmed that these changes are significant, reflecting infection-driven modulation of phenolic metabolism across citrus cultivars.

Table. Differential accumulation of polyphenolic compounds in citrus cultivars between healthy and *C*Las infected citrus samples.

Metabolite	Cultivar	Healthy (µg/g DW, mean ± SD)	Infected (µg/g DW, mean ± SD)	Fold Change (HLB/Healthy)	t-test (p-value)	ANOVA (p-value)
Tryptamine	Kinnow	ND	55.1 ± 4.2	↑ (Induced)	0.002 **	0.010 **
2-(Ethyl)oxybenzylidene acetophenone	Kinnow	ND	207–377 ± 15	↑ (Induced)	0.001 **	0.006 **
Morphinan-4-ol-6,7-dione	Kinnow	ND	315 ± 9.8	↑ (Induced)	0.003 **	0.012 **
3-(3,7-Dimethylocta-2,7-dienyl)-indole	Kinnow	ND	377 ± 21	↑ (Induced)	0.002 **	0.008 **
Oxycodone	Kinnow, Nagpur, Daisy, Valencia Late	55.1 ± 3.1	315 ± 18	↑ (5.7-fold)	<0.001 **	<0.001 **
Hydroxyhydrocodone	Nagpur	120 ± 7.6	ND	↓ (Lost)	0.004 **	0.011 **
Cyclotrisiloxane derivatives	Nagpur, Daisy, Lezben	95 ± 6.2	ND	↓ (Lost)	0.006 **	0.020 *
2-Methoxy-4-vinylphenol	Daisy, Saddis, Valencia Late	30 ± 2.1	145 ± 7.4	↑ (4.8-fold)	<0.001 **	0.003 **
3-Methoxyacetophenone	Daisy, Saddis	18 ± 1.5	98 ± 4.2	↑ (5.4-fold)	<0.001 **	0.002 **
Longifolene	Daisy	ND	65 ± 2.8	↑ (Induced)	0.004 **	0.009 **
Caryophyllene-(I1)	Daisy, Valencia	110 ± 5.5	ND	↓ (Lost)	0.002 **	0.014 **
Acethydrazide derivatives	Saddis	ND	75 ± 3.9	↑ (Induced)	0.008 **	0.017 *
Naphtho[1,2-b]quinoxaline	Valencia	ND	88 ± 6.1	↑ (Induced)	0.005 **	0.015 *
Pancracine	Lezben	ND	72 ± 5.4	↑ (Induced)	0.006 **	0.019 *
N-Methyl-1-adamantaneacetamide	Lezben	ND	65 ± 4.7	↑ (Induced)	0.009 **	0.020 *
6-Methyl-2-(3-nitrophenyl)imidazo[1,2-a]pyridine	Lezben	105 ± 6.2	ND	↓ (Lost)	0.003 **	0.012 **

ND = Not Detected.

*Significance codes: *p < 0.05 (*)*, p < 0.01 (**).

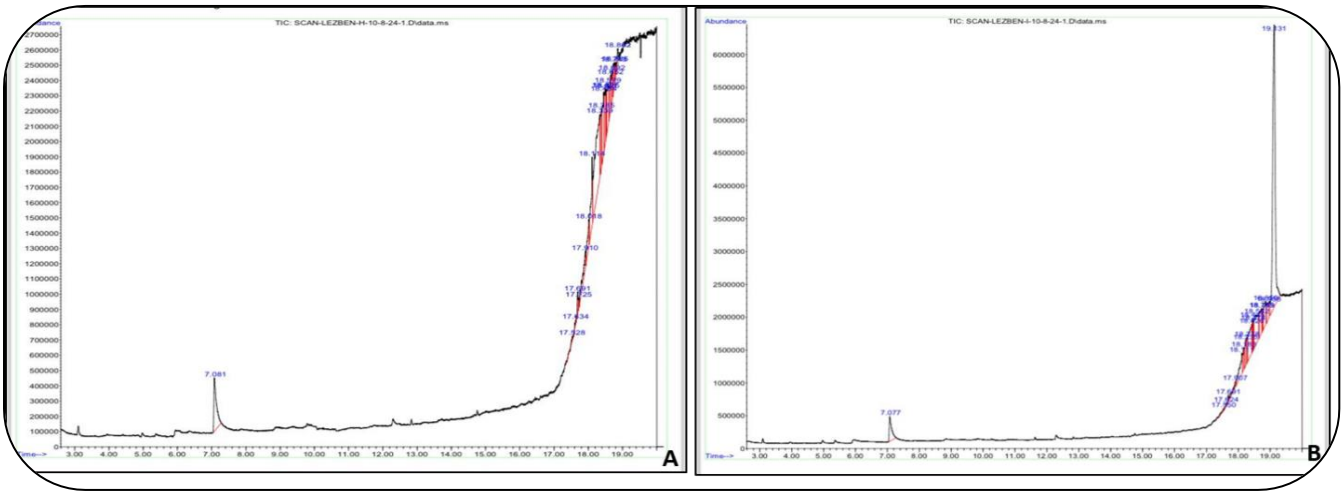

Figure 6. Chromatographic peaks representing phenolic metabolites profile in *Citrus limon* var. Lezben. A) Healthy; B) *C*Las-infected. Legend: Horizontal: time; Vertical: m/z

The dendrogram (Figure 6) showed hierarchical patterns of metabolites, with the vertical axis represented the dissimilarity between them. Shorter vertical lines indicated that the joined metabolites are more similar in their concentration profiles.

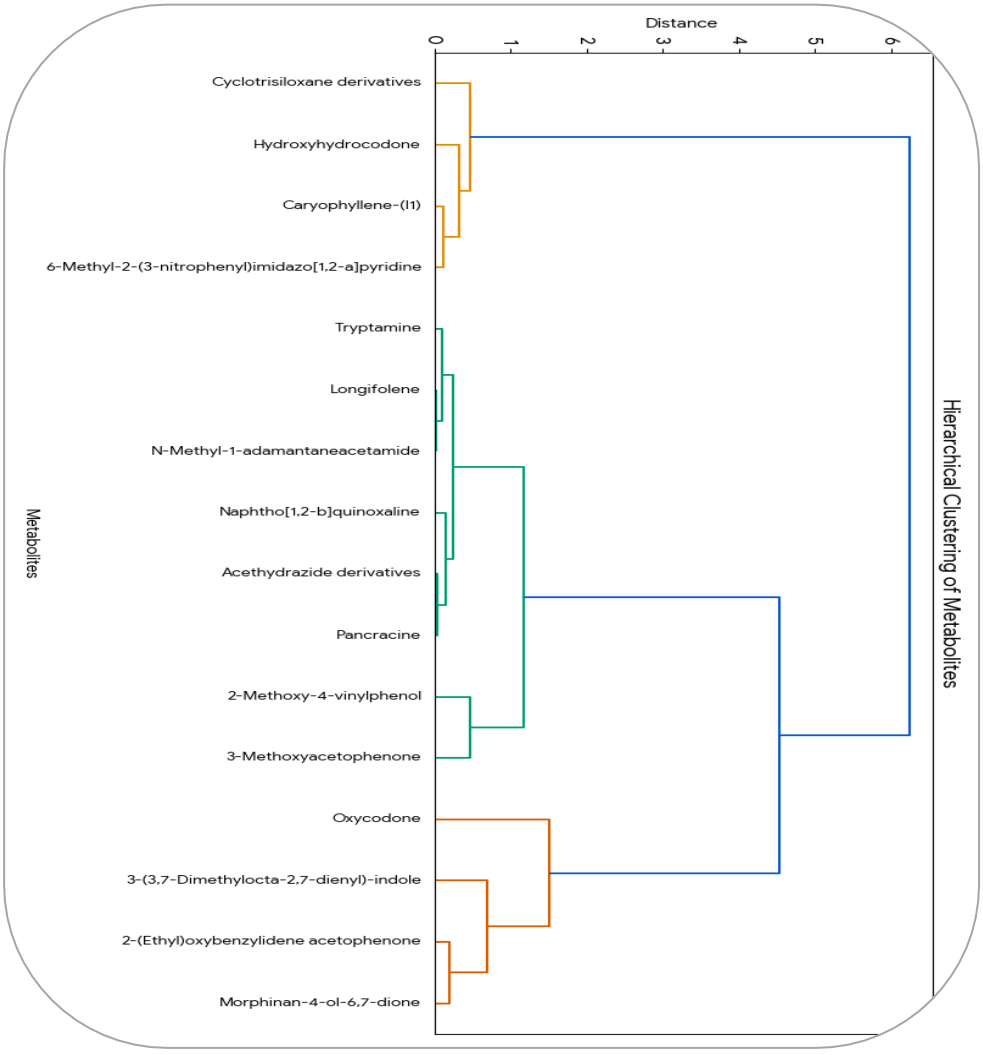

Figure 7. Hierarchical Clustering of phenolic and polyphenolic metabolites identified in citrus cultivars.

Legend: Cluster 1: Metabolites that were not detected (ND) in healthy plants but were present in infected plants, such as Naphtho[1,2-b]quinoxaline, Pancracine, and N-Methyl-1-adamantaneacetamide, are grouped together. Cluster 2: Similarly, a large cluster is formed by other metabolites that were also only induced by infection, including Acethydrazide derivatives, Tryptamine, Longifolene, and Morphinan-4-ol-6,7-dione. Cluster 3: Metabolites that were present in healthy plants but were lost (ND) upon infection, such as Caryophyllene-(I1), Cyclotrisiloxane derivatives, and 6-Methyl-2-(3-nitrophenyl)imidazo[1,2-a]pyridine, are also grouped together. Cluster 4: Metabolites that showed a significant fold change in both healthy and infected samples, like Oxycodone, 2-Methoxy-4-vinylphenol, and 3-Methoxyacetophenone, form a separate group. The dendrogram also highlights that Oxycodone and 2-Methoxy-4-vinylphenol have very similar concentration patterns.

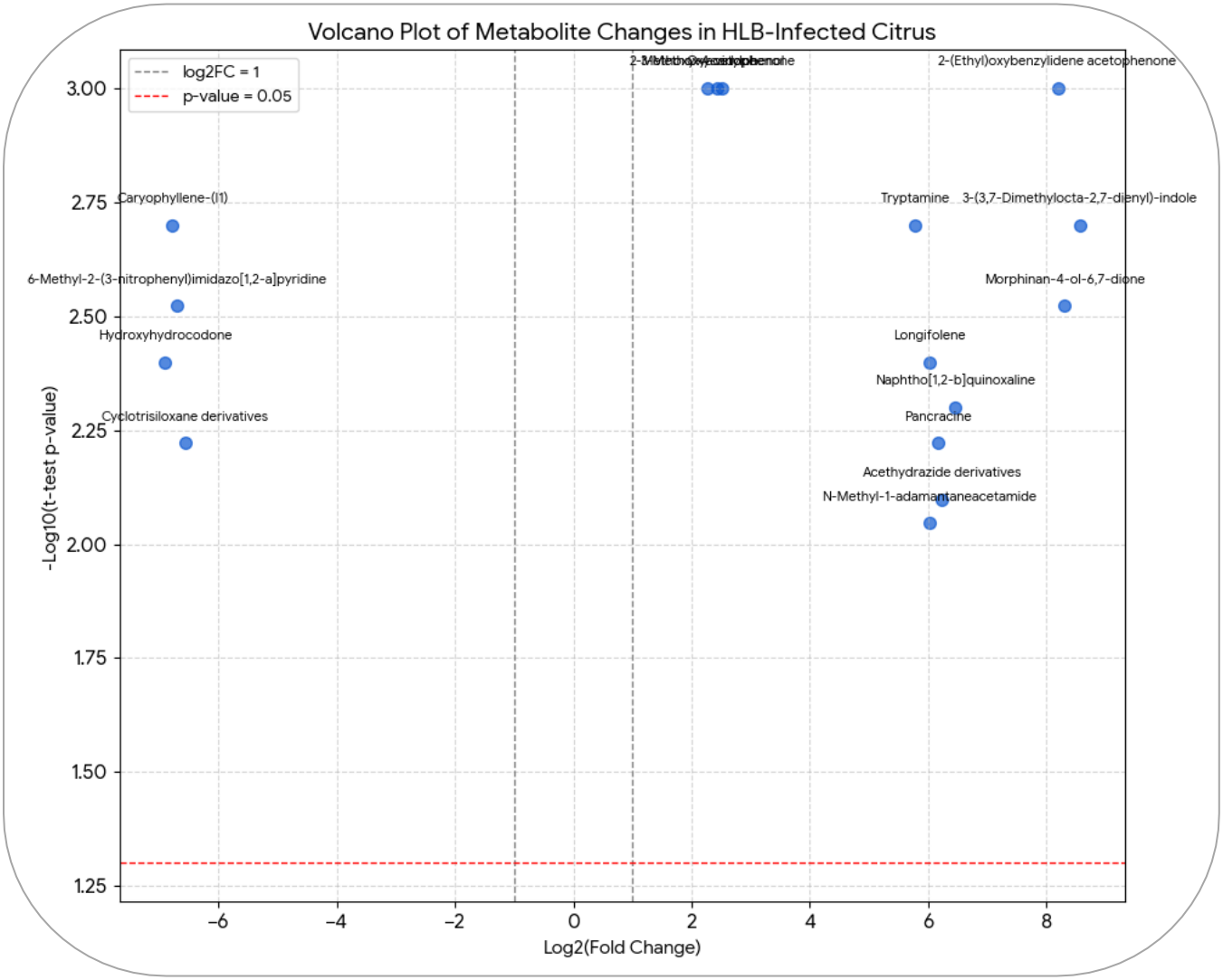

Figure 7. Volcano Plot indicated the clustering of phenolic and polyphenolic metabolites identified in citrus cultivars.

Legend: The plot is divided into four main areas by the significance threshold lines: Top-Right Quadrant: These metabolites are significantly up-regulated in HLB-infected plants, meaning they have a high log2 fold change (more than a 2-fold increase) and a very low p-value (statistically significant). This group includes Oxycodone, 2-Methoxy-4-vinylphenol, and 3-Methoxyacetophenone, as well as several metabolites that were not detected in healthy samples but were induced by the infection. Top-Left Quadrant: These metabolites are significantly down-regulated in HLB-infected plants. They have a high negative log2 fold change (more than a 2-fold decrease) and a low p-value. This group includes Hydroxyhydrocodone, Caryophyllene-(I1), and others

that were lost due to the infection. Bottom Quadrants: Metabolites in this area are not considered statistically significant based on the p-value threshold, even if they show some change in concentration. In this specific plot, all metabolites were statistically significant ($p < 0.05$), so all points appear above the horizontal line.

DISCUSSION

The results from the present study provided compelling evidence that Huanglongbing (HLB) infection in citrus cultivars induces significant alterations in their metabolic and physiological profiles, particularly affecting the presence and abundance of key polyphenolic compounds. The data demonstrated that *C*Las-infected plants exhibited a distinct signature metabolic characterization by the induction of certain metabolites and the loss of others compared to their healthy samples. This pattern is not uniform across all cultivars, suggesting a cultivar-specific response to the pathogen, *Candidatus Liberibacter asiaticus* (*C*Las).

The observed induction of metabolites like Tryptamine, Morphinan-4-ol-6,7-dione, and various unnamed derivatives in *C*Las-infected Kinnow demonstrated the plant's metabolic responses within the *C*Las-Infection. These compounds, often involved in plant defense pathways, are synthesized and accumulated to high levels to combat the systemic infection. For instance, the significant increase in Oxycodone, with a 5.7-fold change in infected Kinnow, points to a robust stress-induced metabolic shift. This was supported by the highly significant p-values (≤0.05), indicating that these changes were not due to random variations but due to the direct effect of the *C*Las-infection. Such a phenomenon was consistent with findings in other host-pathogen interactions where secondary metabolites were upregulated to modulate the host resistance or tolerance (Chen *et al.,* 2022; Kour *et al.,* 2024).

Conversely, this study also revealed the complete loss or significant reduction of certain metabolites, such as Hydroxyhydrocodone in Nagpur and Caryophyllene-(I1) in Daisy and Valencia Late. This loss suggested a potential disruption in normal metabolic pathways. This breakdown of specific metabolites resulted due the disease progression. The downregulation of these metabolites, as evidenced by their absence in *C*Las-infected tissues and the corresponding low p-values, compromised the plant's native defense mechanisms or signal a systemic decline in physiological functions (Hu *et al.,* 202; Pandey *et al.,* 2023; Achrya *et al.,* 2025). The dendrogram further reinforces these findings by clearly clustering metabolites that were induced by *C*Las-infection separately from those that are lost, visually confirming distinct metabolic responses to the disease.

These metabolomic changes were intrinsically linked to the broader physiological health of the plant. The data provided also showed that HLB infection leads to a significant reduction in stem diameter and a more negative water potential in citrus trees compared to healthy controls (Hu *et al.,* 2018; Gaikwad *et al.,* 2025). These physiological markers were indicative of compromised vascular integrity and water transport, which are hallmarks of HLB pathology and lead to plant decline. The combined analysis of metabolomic data with physiological indicators underscores the holistic impact of the disease, where the plant's metabolic distress directly translates into a decline in its physical and structural integrity.

CONCLUSION

In conclusion, these findings collectively demonstrated that HLB infection in citrus cultivars drives a profound metabolic and physiological shift. The data confirmed that the disease induced the synthesis of certain metabolites while suppressing others, with these changes being cultivar-specific and correlating directly with key physiological symptoms. This comprehensive understanding of HLB-induced metabolic pathways provides crucial insights for developing targeted therapeutic strategies and identifying biomarkers for early disease detection.

REFERENCES

Acharya T.P., K.A. Jeffries, K.D. Bowman, B.R. Moser, J.K. Winkler-Moser and C. Dorado. 2025. Investigation of the phytochemical composition of the seeds from four Huanglongbing (HLB) tolerant citrus genotypes and their potential as a biodiesel feedstock. Waste and Biomass Valorization, 1-14.

Ahmad H., N.A. Rajput, M. Atiq, G.A. Kachelo, M. Usman, H. Tariq and M. Wahab. 2024. Detection of *Phytophthora nicotianae* induced citrus gummosis by the loop-mediated isothermal amplification. Pakistan Journal of Botany 56(5): 1-8.

Alquézar B., L. Carmona, S. Bennici, M.P. Miranda, R.B. Bassanezi and L. Peña. 2022. Cultural management of huanglongbing: Current status and ongoing research. Phytopathology, 112(1): 11–25.

Anwar U., M. Mubeen, Y. Iftikhar, M.A. Zeshan, Q. Shakeel, A. Sajid, M. Umer and A. Abbas. 2021. Efficacy of

different fungicides against citrus melanose disease in Sargodha, Pakistan. Pakistan Journal of Phytopathology, 33(1): 67–74.

Bao M., Z. Zheng, X. Sun, J. Chen and X. Deng. 2020. Enhancing PCR capacity to detect '*Candidatus Liberibacter asiaticus*' utilizing whole genome sequence information. Plant Disease, 104(2): 527–532.

Bhatt P., V. Kumar, M.K. Malik and T. Kumar. 2024. Citrus flavonoids: Recent advances and future perspectives on preventing cardiovascular diseases. The Flavonoids: 131–152.

Chen Q., A. Min, S. Luo, J. He, R. Wu, X. Lin, Y. Wang, W. He, Y. Zhang, Y. Lin, M. Li, Y. Zhang, Y. Luo, H. Tang and X. Wang. 2022. Metabolomic analysis revealed distinct physiological responses of leaves and roots to Huanglongbing in a citrus rootstock. International Journal of Molecular Sciences, 23(16): 9242.

Dandlen S.A., J.P. Da Silva, M.G. Miguel, A. Duarte, D.M. Power and N.T. Marques. 2023. Quick decline and stem pitting Citrus tristeza virus isolates induce a distinct metabolomic profile and antioxidant enzyme activity in the phloem sap of two citrus species. Plants, 12(6): 1394.

Djeddour D., C. Pratt, K. Constantine, I. Rwomushana and R. Day. 2021. The Asian citrus greening disease (Huanglongbing): Evidence note on invasiveness and potential economic impacts for East Africa.

Gaikwad P.N., G.S. Sidhu, N.S. Brar, J. Singh, V.Y. Tokala, A. Sharma and P. Manchanda. 2025. Roles of metabolites in fruit maturation, HLB-defense regulation and crosstalk between phytohormone signalling pathways in citrus. Plant Growth Regulation, 1–35.

Gross J., J. Gallinger and L.M. Görg. 2022. Interactions between phloem-restricted bacterial plant pathogens, their vector insects, host plants, and natural enemies, mediated by primary and secondary plant metabolites. Entomologia Generalis, 42(2): 185–215.

Hassan M.U., M.A. Zeshan, Y. Iftikhar, M.U. Ghani, M.A. Shabbir, S. Bashir, S. Saleem and K. Ambreen. 2025. Investigation of citrus melanose disease in Silanwali, Sargodha (Pakistan). Pakistan Journal of Phytopathology 37(1).

Herranz M.C., J.A. Navarro, A. Locascio, L. Peña, P. Manzanares, J.F. Marcos and V. Pallás. 2024. Comparative metabolomic analysis of the phloem sap of nine citrus relatives with different degrees of susceptibility to Huanglongbing disease. European Journal of Plant Pathology, 170(3): 463–478.

Hosseinzadeh S. and M. Heck. 2023. Variations on a theme: Factors regulating interaction between Diaphorina citri and '*Candidatus Liberibacter asiaticus*' vector and pathogen of citrus huanglongbing. Current Opinion in Insect Science, 56: 101025.

Hu B., M.J. Rao, X. Deng, S.S. Pandey, C. Hendrich, F. Ding and Q. Xu. 2021. Molecular signatures between citrus and *Candidatus Liberibacter asiaticus*. PLoS Pathogens, 17(12): e1010071.

Hu J., J. Jiang and N. Wang. 2018. Control of citrus Huanglongbing via trunk injection of plant defense activators and antibiotics. Phytopathology, 108(2): 186–195.

Huang X., W. Ma, W. Wang, Y. Feng, T. Lamichhane, J. Xu and N. Wang. 2025. Mechanistic dissection of *Candidatus Liberibacter* triggered chronic immune disease. bioRxiv.

Killiny N. 2019. Collection of the phloem sap, pros and cons. Plant Signaling and Behavior, 14(8): 1618181.

Kour S., N. Sharma, A. Khajuria, D. Kumar and P. Ohri. 2024. Elucidating the role of flavonoids in countering the effect of biotic stress in plants. Plant Phenolics in Biotic Stress Management: 121–148.

Li B., S. Wang, Y. Zhang and D. Qiu. 2020. Acid soil improvement enhances disease tolerance in citrus infected by *Candidatus Liberibacter asiaticus*. International Journal of Molecular Sciences, 21(10): 3614.

Li M. and Z. Nangong. 2022. Precision trunk injection technology for treatment of huanglongbing (HLB)-affected citrus trees—a review. Journal of Plant Diseases and Protection, 129(1): 15–34.

Limayem A., E.M. Martin and S. Shankar. 2024. Study on the citrus greening disease: Current challenges and novel therapies. Microbial Pathogenesis, 192: 106688.

Monteiro F., S.S. Shetty, V.V. Shetty, D.P. Shetty and P. Patil. 2022. Phytochemical profiling, total flavonoid, total phenolic content and *in-vitro* antioxidant evaluation of Citrus maxima extract. Biomedicine, 42(5): 912–919.

Palangasinghe P.C., W.K. Liyanage, M.P. Wickramasinghe,

H.R. Palangasinghe, H.C. Shih, M.S. Shiao and Y.C. Chiang. 2024. Reviews on Asian citrus species: Exploring traditional uses, biochemistry, conservation, and disease resistance. Ecological Genetics and Genomics, 32: 100269.

Pandey S.S., J. Xu, D.S. Achor, J. Li and N. Wang. 2023. Microscopic and transcriptomic analyses of early events triggered by '*Candidatus Liberibacter asiaticus*' in young flushes of Huanglongbing-positive citrus trees. Phytopathology, 113(6): 985–997.

Pérez-Hedo M., M.S. Hoddle, F. Alferez, B. Lochy, G.A. Beattie, S. Chakravarty and A. Urbaneja. 2025. Global strategies to manage huanglongbing (HLB) and its vectors: Insights and implications for the Mediterranean region. Entomologia Generalis, 1–16.

Serag A., M.A. Salem, S. Gong, J.L. Wu and M.A. Farag. 2023. Decoding metabolic reprogramming in plants under pathogen attacks: A comprehensive review of emerging metabolomics technologies to maximize their applications. Metabolites, 13(3): 424.

Sultan A., M. Atiq, N.A. Rajput, S. Iftekhar, M.M. Jahangir, A. Latif, M. Saleem, M. Asghar, Z. Haider, M. Umair and M.J. Matloob. 2025. Enhancing citrus resistance against Huanglongbing using plant activators and copper acetate. Phytopathogen omics and Disease Control, 4(1): 39–44.

War A.F., S.A. Nanda, I. Bashir, S. Rehmaan, I.A. Sheergojri, I.U. Rehman and I. Rashid. 2024. Plant phenolics role in bacterial disease stress management in plants. Plant Phenolics in Biotic Stress Management, 217–241.

Welker S., M. Pierre, J.P. Santiago, M. Dutt, C. Vincent and A. Levy. 2022. Phloem transport limitation in Huanglongbing-affected sweet orange is dependent on phloem-limited bacteria and callose. Tree Physiology, 42(2): 379–390.

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