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Antimicrobial volatile compounds of healthy mandarin orange imparting resistance to *Candidatus* Liberibacter asiaticus

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Abstract

Citrus greening disease (HLB), caused by "*Candidatus* Liberibacter asiaticus" (CLas), threatens the citrus industry. Metabolite profiling of pathogen-host interactions revealed several fatty acids, monoterpenoids, and fatty alcohols. These compounds have antimicrobial properties and activate the defense mechanism in citrus spp. Gas chromatography-mass spectrometry (GC-MS) profiles the volatiles of CLas-infected and healthy *C. reticulata* leaf samples. Two different patterns of volatiles were eluted from the extract of healthy and CLas-infected mandarin orange leaves. Major volatiles in healthy mandarin orange leaves included p-vinyl guaiacol (9.76%), chinic acid (5.21%), and beta citronellol (2.68%). However, CLas-infected leaves had a distinct volatile pattern compared to healthy ones. CLas-infected leaves exhibit the compounds of quercinitol (5.99%), hexose (1.19%), D-arabitol (0.89%), and scyllite (0.6%). The study showed that robust mandarin orange leaves have more beta-citronellol and P-vinylguaiacol than other profiles. In addition to exhibiting active antibacterial activity against *Proteus vulgaris* and *Escherichia coli* as described by earlier research, P-vinylguaiacol has been reported to have mild inhibitory effects on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* from *P. laevigata* subsp. *angustifolia* fruits. Citrus trees have oil glands in their phloem tissues, midribs and veins, therefore volatile compounds may directly affect the CLas disease and its vectors (*Diaphornia citri* and *Trioza erytreae*). Linalool changes in the volatile profile, a marker of CLas infection in citrus can benefit from cumulative volatile profiling in popular kinds, seasons and locations.

1. Introduction

Huanglongbing (HLB), also known as citrus greening disease, is one of the economically significant diseases of the citrus industry and is responsible for damaging more than 60 million citrus trees worldwide (Halbert *et al*., 2004; Bove, 2006). *Candidatus* Liberibacter asiaticus (CLas)is a gram-negative, phloem-limited bacteria causing huanglongbing disease in mandarin orange. It comes under alpha-proteobacteria based on its place of origin and 16S rRNA. The asian citrus psyllid (*Diaphorina citri*, or ACP) insect and grafting contaminated material are the two main vectors for spreading the bacterium (Gottwald *et al*., 2007; Lopes *et al*., 2009). Early stages of CLas infection have no noticeable effects on the fruit, leaf or tree's external appearance. Polymerase chain reaction (PCR) is the gold-standard for detecting bacteria since it can identify infections before the pathogen, manifest externally either in leaf or fruit. The HLB disease emerges once the tree eventually becomes symptomatic/ HLB affected which can take several years resulting in discoloured leaves, misshapen and bitter fruit, 80% yield reductions and ultimately early tree death. HLB is considered as the most serious citrus disease in the world and there is no known management practices as of now (Cevallos-Cevallos *et al*., 2012; Bove, 2006). Prevention of disease spread is primarily focused toward controlling the psyllid populations through trapping as well as pesticide application. Furthermore, it has been demonstrated that the implementation of increased nutrient programmes has no appreciable impact on enhancing tree health, fruit quality and yield (Cevallos-Cevallos *et al*., 2012; Folimonova *et al*., 2009; Lopes *et al*., 2009). In India, majority of citrus citrus cultivation areas are infected with HLB. Since there has no proper management strategy of controlling haunglongbing in Tamil Nadu still now, the present objectives such as detection of CLas through PCR identification of brix rate and analysis of volatile compounds

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through GC-MS will bring a proper understanding of the HLB and management strategies to control the CLas. Despite the fact that the disease has been reported from all of India's citrus growing states. DNA was extracted from the samples collected at Palani hills (Kanalkadu) in Tamil Nadu and the pathogen was confirmed with specific primers O11 and OI2C primers before analysing GC-MS (Figure 1 and Figure 2). Samples from a total of 10 healthy and 10 HLB free mandarin orange were collected originating from Kanalkadu, Dindigul district and among them, five best trees samples showing proper HLB symptoms and five trees showing no HLB symptoms, selected for further studies such as PCR detection, Brix rate and GC-MS analysis.Together, these findings show that CLas infection causes a significant metabolic response in many mandarin orange trees in Tamil Nadu, raising the possibility of developing metabolite based biomarkers for CLas infection. After being attacked by a pathogen, plants launch a variety of protective measures to prevent additional damage (Schilmiller and Howe, 2005; Durrant and Dong, 2004; Ryals *et al*., 1996; Kim *et al*., 2009) including alterations in gene expression, plant communication, and other biochemical and physiological changes (Durrant and Dong, 2004; Ryals *et al*., 1996). These alterations can be observed throughout the entire plant and offer hints for comprehending how a disease affects its host plant. Gaining an overview of the changes that take place in a plant following injury which can be accomplished by using metabolomics, that describes the entire set of metabolites produced in an organism. Using GC-MS based metabolomics, macerated leaves from healthy (n = 9) and HLB infected mandarin orange (*Citrus reticulata* Blanco) leaves (n = 9) were compared in order to determine the impact of CLas infection on the citrus metabolome (Table 1 and Figure 9). In the fields of the mandarin orange orchard in Kanalkadu, Palani Hills, Dindigul District, Tamil Nadu, differences in the concentration of several metabolites, such as beta-citronellol, vanillic acid, diethylene glycol, quinic acid and n-hexadecanoic acid produced in healthy mandarin orange leaves were noticed.

The presence of amino acids, organic acids, sugars and small amounts of free fatty acids were found in citrus phloem sap according to the composition analysis. The repressors of the type III genes of plant pathogens, fatty acids have been proposed to have a role in plant defence mechanism (Xiao *et al*., 2004). The absence of a type III secretion system in *Candidatus* Liberibacter asiaticus (Duan *et al*., 2009) raises the possibility of a more widespread involvement for fatty acids, such as an antioxidant function in citrus defence. Although, fatty acids play important roles in pathogen defense in plants which alter biochemical pathways relating to fatty acid metabolism under HLB have not been thoroughly studied. Palmitoleic acid (C16:1) was found to directly inhibit pathogenic growth, enhancing the host resistance to biotic stress (Xing and Chin, 2000). According to reports, oxylipins and linoleic acid (C18:2) have a significant role in plants immune systems react to microbes (Novitskaya *et al*., 2002). This suggests that a lack of fatty acid intracellular responses can lead to a reduction in immunological function, potentially accelerating the onset of pathology (Suh *et al*., 2018). Systemic infection of Citrus spp. with the pathogen CLas leads to alterations in the organic acids and essential fatty acids distinctive metabolite makeup. There is evidence that these substances play a part in signalling and activating plant defences. These chemicals may be employed as a low cost assay for the field diagnosis of HLB disease after they have been well described and associated with the CLas infection. This can be accomplished using targeted metabolomics and selective metabolite analysis (Cevallos Cevallos *et al*., 2011). Similarly, GC-MS profiling of headspace metabolites in mangoes is used to differentiate between anthracnose and stem end rot infections in mangoes. Target metabolites can also distinguish the HLB between citrus varietals (Folimonova *et al*., 2009; Moalemiyan *et al*., 2007).

Additionally, prior research revealed that phenolic structured essential oil components, including thymol, have the most substantial antibacterial effects. Healthy Citrus spp. contain significant concentrations of monoterpene alcohols, diterpene alcohols, thymol, and its precursors, which are the main causes of their susceptibility to the CLas infection. It is hypothesised that finding important metabolites that are particular to the interaction between citrus and HLB comparing them to HLB tolerance or resistance may lead to the discovery of new HLB biomarkers. Therefore, the purpose of this work was to characterise the signature metabolite for the pre- diagnosis of the HLB sickness and to analyse the volatile profiles. Monoterpene alcohols, diterpene alcohols and their precursors are present in large amounts in healthy Citrus spp. are the primary causes of their tolerance to the CLas infection .The goal of the current study was to characterise five HLB isolates from Tamil Nadu using 16S rRNA genomic area analysis, Brix rate and to pinpoint the metabolites that are crucial for controlling Huanglongbing.

2. Materials and Methods

2.1 Sample size and experiment layout

Fourteen years old Nagpur mandarin orange (*Citrus reticulate* Blanco) trees that were planted at high density planting at the spacing of each tree is 6 × 6 metres (130 plants per acre) plot was selected. For the analysis, physiologically mature leaves and fruits that show HLB illness were collected from Kanalkadu hamlet in the Tamil Nadu, India region of Dindigul district (10.2968° N; 77.7088° E) during march 2021.

Each tree was separated into four quadrants (north, south, east, and west), and the fruits from each quadrant were collected and pooled with fully expanded leaves still attached. Healthy or asymptomatic leaves as well as those with typical HLB signs were gathered and appropriately labelled (Batool *et al*., 2007). After the collection of leaf samples, 1 g of leaf midrib sample was taken for DNA extraction and 300 milligrams of leaf midrib samples were taken for GC-MS extraction and 1 ml of CLas affected mandarin orange fruit juice for checking Brix rate.

2.2 Plant components

Nagpur mandarin variety samples were brought from the Kanalkadu region and polymerase chain reaction (PCR) was used to confirm the presence of CLas bacteria in symptomatic and asymptomatic leaf samples by following the established standard procedures (Li *et al*., 2006). To detect CLas and to perform GC-MS analysis, mandarin orange leaves with chlorosis and blotching signs were gathered specifically.To look into the brix rate, mandarin orange fruits with symptoms, asymptomatic fruits and healthy fruits were gathered. Fruits can be identified from one another depending on their shape and size. Prior to determining the brix rate, PCR was used to confirm and distinguish between CLas free and CLas positive in both leaves and fruits. However, the majority of asymptomatic leaves and fruits tested negative for CLas, despite the fact that morphological appearance alone could not identify the CLas invasion in leaves

from other invasions. As a result, a test using both healthy and CLas positive samples was conducted.. Five tree samples of each CLas affected mandarin oranges and healthy among all observed trees were gathered based on the peculiar CLas symptoms and leaf samples were pooled, DNA extraction and PCR was carried out. Already derived CLas affected mandarin orangeleaf samples were used as control for confirmation of CLas. All samples came from 14-year-old, fully grown trees. In each block, samples were taken from three different places of the trees. 15-20 leaves from each tree were included and pooled for each tree and kept at -20°C before DNA extraction. From March to April 2019, samples of mandarin orange leaves were observed in commercial orchards in Palani Hills, Kanalkadu and further work was conducted.

2.3 DNA extraction, PCR and evaluation of fruits brix rate

Samples were transported from the Kanalkadu village (Palani hills) and kept at –80°C. For initial confirmation of pathogen (CLas) in the samples, CTAB method was used to extract DNA (Doyle, 1991). Using a sterile surgical blade, 1 g of mandarin orange midribs were separated, and the midribs were then cut into little bits and macerated by adding liquid nitrogen to a pestle and mortar. In a new Eppendorf tube, 2 ml of CTAB buffer was added into the macerated sample and incubated in a water bath for 20 min at 65°C (Scientech, India). The samples were inverted once every 30 sec each minute and centrifuge the samples at 10,000 rpm for 15 min. The supernatant was collected, and the equal amount of 24:1 chloroform : isoamyl alcohol was added. Then, the centrifuge was then set to 10,000 rpm for 10 min. A fresh tube was taken and transferred the aqueous phase and the procedure was repeated by adding an equal amount of 24:1 chloroform : isoamyl alcohol The aqueous phase was collected in a fresh tube and an equivalent volume of ice cold isopropanol was added. This tube was then maintained at 20°C for 3 h . The material was centrifuged at 10,000 rmp for 20 min. The DNA pellet was collected and the supernatant was discarded. 70 per cent ethanol was added and centrifuged at 5000 rpm for 5 min. After an ethanol wash, air dried the pellet by discarding the supernatant. For later usage, the samples were added to 50 µl of sterile water and kept at – 20°C. (Figure 1). The amount of the isolated DNA was calculated by measuring the O.D. values at 260 nm and 280 nm using a NanoDrop spectrophotometer (NanoDrop, TNAU). DNA samples with calorimetric readings between 1.8 and 2.0 were collected for PCR analysis. Primers set O11F/OI2cR (GCG CGT ATG CAA TAC GAG CGG CA) / (GCC TCG CGA CTT CGC AAC CCA T) (Ahmad *et al*., 2009; Jagoueix *et al*., 1996) targeting partial, 16SrDNA were used in a conventional PCR (Eurofins) amplification (Figure 2) (most specific region of the CLas genome). The reaction mixture was prepared for a 25 µl volume using 0.5 µl of dNTPs (10 mM), 5 µl of 10x buffers, 2.0 µl of forward and reverse primers (10 mM), 5 µl of DNA template (100-200 ng/l), and 0.3 µl Taq polymerase (5 units/l, GeneiTM) with the remaining volume being made up with nuclease free water. The thermal cycle consisted of a cycle at 95°C lasting 2 min, followed by 35 cycles at 95°C lasting 40 sec, a cycle at 60.5°C lasting 1 min, a cycle at 72°C lasting 1 min, and a 72°C extension lasting 10 min (Xie *et al*., 2018). The amplification product was analysed in a Tris-acetate EDTA buffered 1.2% agarose gel containing ethidium bromide. The samples that tested positive were verified as having HLB infection and the ones that tested negative were verified as being in good health. The amplicons were examined under U.V. light in a gel documentation system. The metabolites found in these trees were subsequently identified using GC-MS (Gas chromatography and Mass spectrometry). The Brix rate was evaluated through Hand refractometer (Emra) (Table 1). Among the ten collected leaf and fruit samples, five samples showed positive for CLas and and the remaining showed negative for CLas. The mandarin orange tree which showed more symptoms of CLas and less Brix rate and the mandarin orange tree which showed high Brix rate and negative for CLas was considered as healthy mandarin orange and were taken for identification of volatile compounds through GC-MS.

2.4 Extracting volatiles from the leaves of the mandarin orange

Based on PCR results, samples of mandarin orange (*Citrus reticulata* Blanco) (Figure 1) were differentiated as positive and negative. After that, GC-MS extraction was carried out by following the protocol given by Lisec *et al*., (2006) by following three steps such as extraction, fractionation and derivatization, volatiles extract were prepared for both CL as positive and negative CL as leaf samples.

Extraction : 300 milligram macerated leaf sample was transferred to two ml microcentrifuge tubes in which 1.4 ml of 100 per cent methanol was added to stop the enzyme activity, and the tubes were vortexed. A 50 µl of ribitol (0.2 mg/ml of water) was added as an internal reference. Then 50 µl of deionized water was added and the tubes were vortexed and the pH was checked. The pH at this stage was expected to be around 6. The tubes were incubated at 70°C with continuous shaking with 900 rpm for 10 min. After 1 min of incubation, the tubes were opened and the incubation was continued for the remaining 14 min. After 15 min of incubation, The tubes were centrifuged at 12,500 rpm at 11000 G rotor value (RCF) for 20 min at 4°C. The supernatant was transferred to Ultrafree CL 0.22 µl filter tubes and 1.4 ml of water was added to these tubes. 750 µl of chloroform was added and vortexed to the pellet left behind in microcentrifuge tubes. The tubes were incubated at 37°C for 10 min with continuous shaking. The tubes were centrifuged at 3000 rpm for 20 min at 4°C and the supernatant was transferred to the same ultra free CL 0.22 µl filter tubes in which the previous supernatant was added. The tubes were vortexed and centrifuged at 4000 rpm for 15 min at 4°C.

Fractionation: The final concentration described in the above procedure led to the separation of two phases: the upper polar phase and lower non-polar phase. One ml upper polar phase was transferred to new microcentrifuge tubes and the remaining 1.8 ml of polar phase were stored in –80°C for future use and the tubes containing one ml of polar phase were sealed with parafilm and holes were punched in parafilm. Later the samples were allowed to dry in concentrator (Eppendorf) at 30°C.

Derivatization: After the samples were concentrated, A total of 80 µl of methoxamine hydrochloride (20 mg/ml Pyridine) was added to the dried fraction of mobile phase (Fractionation) and the tubes were incubated at 30°C for 90 min with continuous shaking. An 80 µl of MSTFA was added and again the tubes were set at 37°C for 30 min. After incubation, the tubes were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was transferred to micro glass tubes. The derivatized samples are quickly injected into the GC-MS instrument (Shimadzu) to derive metabolites accurately.

2.5 Analysis of mandarin orange leaves' volatile profiles

Using a Shimadzu gas chromatography machine with a mass detector, the volatile components of both healthy and diseased mandarin orange leaf samples were analysed. The samples were separated using an Agilent DB-5 ms column, 60 m 0.25 mm I.D. a film thickness of 0.25 mm. Using a Hamilton Microliter (Reno, NV) syringe, 0.3 mL of the derivatized extract was injected into the GC-MS. Before each use, the syringe was cleaned with hexane ten times. The settings for the G.C. technique were as follows: a 250°C injector temperature, a 70°C beginning oven temperature, a 10°C min^{-1} ramp to 310°C, and a 5 min hold at 310°C. At a rate of 1 ml min^{-1}, ultrapure hydrogen was used as the carrier gas. The total ion current was measured for a mass range of 50-650 AMU, while the M.S. was adjusted to its highest level of sensitivity in electron impact mode, positive polarity. At 318°C, the GC-MS interface was set. With a scan frequency of 4 s and a total G.C. time of 8 min, the scan was captured (24 min). 1.0 ml aliquots of the methanol extract were loaded into the chromatograph. A computer driven technique was used to identify the main ingredients, and after that, the mass spectrum of the study was compared to the National Institute of Standards and Technology (NIST) library (Version. 2.0, year 2005). Turbo mass 5.1 was the programme used for gas chromatography-mass spectrometry (GC-MS), Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Lawley Road, Coimbatore, Tamil Nadu, India, is where this work has been done (Figures 5, 6 and 8).

2.6 Statistical analysis

The design of the experiment and the statistical analysis was performed using OPSTAT and DMRT (Duncan Multiple Range Test) and analysed through SPSS software package. NIST LIBRARY (AMDIS) software was used to conduct a comparative examination of volatile compounds and structures. Metabo Analyst 5.0 (http://www.metaboanalyst.ca/) was used to carry out the Euclidean pathway for representation of volatile compounds.

3. Results

The following volatile compounds were analysed in CLas infected mandarin oranges through GC-MS

1,7-octadiene-3,6-diol, 2,6-dimethyl-thiazolidine, 2-(2-furyl)-piperidin-2-one-5-carboxylic acid, 6-dimethyl (trimethylsilyl) silyloxytetradecane. 5,6-dihydride, 2-methoxy-4-vinylphenol, 3-buten-2-one, 3-trimethylsilyloxy, benzeneacetonitrile, 4-hydroxy, quinic acid, d-talonic acid lactone, isoamyl alcohol, 4,5-dihydroxy-6-hydroxymethyl-oxepan-3-one, 3-hydroxy-4-methoxycinnamic acid, methyl gal (Figures 3, 5 and 6).

The following volatile compounds were analysed in healthy mandarin oranges through GC-MS

pyridine, 1-(2-hydrazino-2-oxoethyl)-, 2-pyridinecarboxyaldehyde, diethylene glycol, benzoic acid, 1,7-octadiene-3,6-diol, 2,6-dimethyl, glycerol, beta-citronellol, trifluoroacetate, 2-methoxy-4-vinylphenol, acetin, 1,2,3- (Figures 4 and6).

Benzeneacetonitrile, 4-hydroxy, quinic acid, 4,5-dihydroxy-6-hydroxymethyl-oxepan-3-one, and n-hexadecanoic acid are the components that are similar in both healthy and infected mandarin oranges. 1,7-octadiene-3,6-diol, 2,6-dimethyl-, 2-methoxy-4-vinylphenol, 2,6-dihydroxy, 2-met (Figure 7, Table 2 and Table 3).

The monoterpenoid and fatty acid volatiles predominated in the GC-MS chromatogram of the methanol extract of mandarin orange leaves. The compounds peak area per cent, retention time, molecular weight, structure, and biological activity were noted. Utilizing the NIST collection, mass spectra were used to identify volatiles.

This study showed that an n-methanol extract of healthy mandarin orange leaves eluted 25 volatile compounds. Beta citronellol, a monoterpene alcohol molecule with a peak area of 2.68 per cent was the primary substance found in the chromatogram. In addition to β -citronellol substances found such as 4,5-dihydroxy-6-hydroxy methyl-oxepan-3-one (26.08%). Quinic acid (5.21%), a benzyl compound, 2-methoxy-4-vinylphenol (9.76%), and diethylene glycol (1.29%), a dialkyl ether are also present. (Figure 6 and Table 5).

Mandarin orange leaves infected with HLB showed a completely new pattern of volatiles, consisting of 25 chemical compounds. Quercinitol (Scyllo inositol), cyclo hexanol with a peak area percentage of 5.99%, D-galactose (1.19%), which is a hexose group, Arabinitol with a peak area of (0.89%), which is a sugar alcohols and Myo inositol (0.6%), which is a cyclohexanols are major signs of the infection (sugars, $C_6H_{12}O_6$) in the infected plants (Table 4 and Figure 7). The dominance of monoterpenes was demonstrated by another prominent fraction volatile profile of the healthy mandarin orange leaves. In flowers, fruits and young leaves, monoterpenol oxidative metabolism normally regulates the production of volatiles.

Table 1: PCR detection and Brix value of healthy and infected mandarin orange leaf and fruit

Healthy samples (5)	PCR detection mandarin orange samples (5)	HLB infected	PCR detection	Location	Altitude	Brix rate (healthy)	Brix rate (infected)
Mandarin orange tree 1	Positive	mandarin orange tree 1	Negative	Kanalkadu, Palani hills	1098 metres	6.9^{a}	5.05^{e}
mandarin orange tree 2	Positive	mandarin orange tree 2	Negative	Kanalkadu, Palani hills	1098 metres	6.8^{bc}	5.20^{c}
Mandarin orange tree 3	Positive	mandarin orange tree 3	Negative	Kanalkadu, Palani hills	1098 metres	6.5^{cd}	5.40^{a}
Mandarin orange tree 4	Positive	mandarin orange tree 4	Negative	Kanalkadu, Palani hills	1098 metres	6.4^{e}	5.20^{d}
Mandarin orange tree 5	Positive	Mandarin orange tree 5	Negative	Kanalkadu, Palani hills	1098 metres	6.6^{f}	5.30^{b}
C.D.						0.331	0.206
SE(m)						0.100	0.062
SE(d)						0.141	0.088
C.V.						2.604	2.062

*The treatment means are compared using Duncan's Multiple Range Test (DMRT).
In a column, means followed by a common letter (s) are not significantly different (p=0.05).

Table 2: The GC-MS compounds common in both (H and I) that were derived from CLas infected mandarin orange total ion chromatogram at different area percentage

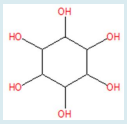
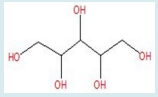
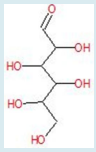
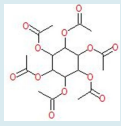
Identity from NIST library	Compound name	Retention time	Area	Area%	Formula	Molecular weight
2,6-Dimethylocta-1,7-dien-3, 6-diol	1,7-Octadiene-3,6-diol, 2,6-dimethyl	8.658	1289599	0.29	$C_{10}H_{18}O_6$	170
p-Vinylguaiacol	2-Methoxy-4-vinyl phenol	9.34	9348794	2.09	$C_9H_{10}O_2$	150
acetonitrile	Benzeneacetonitrile, 4-hydroxy	11.606	3339512	0.15	C_8H_7N	117
Chinic acid	Quinic acid	13.246	9619793	2.15	$C_7H_{12}O_6$	192
1,6-Anhydro-5-deoxy-5-(hydroxymethyl)hex-2-ulose	4,5-Dihydroxy-6-hydroxymethyl -oxepan-3-one	15.694	130735053	29.23	$C_7H_{12}O_5$	176
Palmitic acid	n-Hexadecanoic acid	17.11	2967535	0.66	$C_{16}H_{32}O_2$	256

Table 3: The GC-MS compounds common in both (H and I) that were derived from healthy mandarin orange leaves total ion chromatogram at different area percentage

Identity from NIST library	Compound name	Retention time	Area	Area%	Formula	Molecular weight
2,6-Dimethylocta-1,7-dien-3, 6-diol	1,7-Octadiene-3,6-diol, 2,6 -dimethyl	8.656	4465087	1.5	C10H18O2	170
p-Vinylguaiacol	2-Methoxy-4-vinyl phenol	9.339	29034610	9.76	$C_9H_{10}O_2$	150
acetonitrile	Benzeneacetonitrile, 4-hydroxy	11.658	11300622	3.8	C_8H_7N	117
Chinic acid	Quinic acid	13.267	15506658	5.21	$C_7H_{12}O_6$	192
1,6-Anhydro-5-deoxy-5-(hydroxymethyl)hex-2-ulose	4,5-Dihydroxy-6-hydroxy methyl-oxepan-3-one	15.3 75	77585312	26.09	$C_7H_{12}O_5$	176
Palmitic acid	n-Hexadecanoic acid	17.099	1871935	0.63	$C_{16}H_{32}O_2$	256

*All the common samples that were present in healthy and HLB affected mandarin orange came in same retention time, this clearly shows that the compounds which are showing in both TIC were authenticated.

Table 4: GC-MS analysis of unique volatile compounds in CLas infected mandarin orange leaves (as per literature cited)

Identity from NIST library	Compound name	Retention time	Area	Area%	Formula	Molecular weight	Structure
Scyllite	**Myoinositol**	17.75	2701637	0.6	$C_6H_{12}O_6$	180	
D-Arabitol	**Arabinitol**	16.538	3967021	0.89	$C_5H_{12}O_5$	152	
Hexose	**D-Galactose**	11.042	5304553	1.19	$C_6H_{12}O_6$	180	
Quercinitol	**Scyllo-Inositol**	18.205	26783266	5.99	$C_6H_{12}O_6$	180	

The compound myoinositol produced at 17.75 retention time, whereas the area per cent/amount at at 0.6 per cent. The compound arabinitol produced the peak at retention time 16.538, and 10.89 area per cent, whereas the D-galactose at 11.042 RT time and scyllo inositol at 18.205 RT and 5.99 area per cent

Table 5: GC-MS analysis of unique antimicrobial volatile compounds in CLas infected mandarin orange leaves (as per literature cited)

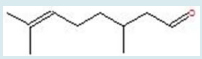
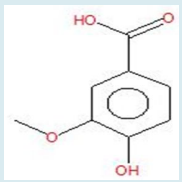
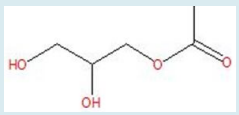
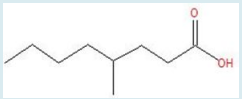
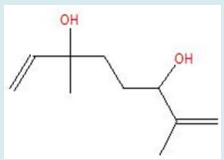
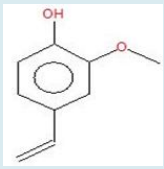
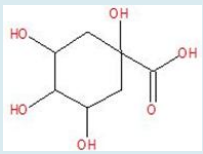
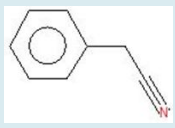
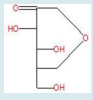
Compound name	Retention time	Area	Area%	Formula	Molecular weight	Structure
beta-citronellol	9.031	7956424	2.68	$C_{10}H_{18}$	138	
Vanillic acid	32.601	1764363	0.59	$C_8H_8O_4$	168	
Diethylene glycol	8.252	3839542	1.29	$C_4H_{10}O_3$	106	
Acetin	9.691	946639	0.32	$C_5H_{10}O_4$	134	
4-Methyloctanoic acid	10.985	1649818	0.55	C9H18O2	158	
1,7-Octadiene 3,6-diol, 2,6-dimethyl	8.656	4465087	1.5	$C_{10}H_{18}O_2$	170	
2-Methoxy-4-vinylphenol	9.339	29034610	9.76	C9H10O2	150	
Quinic acid	13.267	15506658	5.21	$C_7H_{12}O_6$	192	
Benzeneacetonitrile, 4-hydroxy	11.658	11300622	3.8	C_8H_7N	117	
4,5-Dihydroxy-6-hydroxy methyl-oxepan-3-one	15.692	77585312	26.09	$C_7H_{12}O_5$	176	
n-Hexadecanoic acid	17.099	1871935	0.63	$C_{16}H_{32}O_2$	256	

Table 6: GC-MS analysis showing total functional groups obtained from both healthy and HLB affected mandarin orange leaf samples

Unique compounds	Amides	alcohol	ethers
Compounds in healthy mandarin orange leaves	1	8	2
Compounds in Infected mandarin orange leaves	Nill	3	1

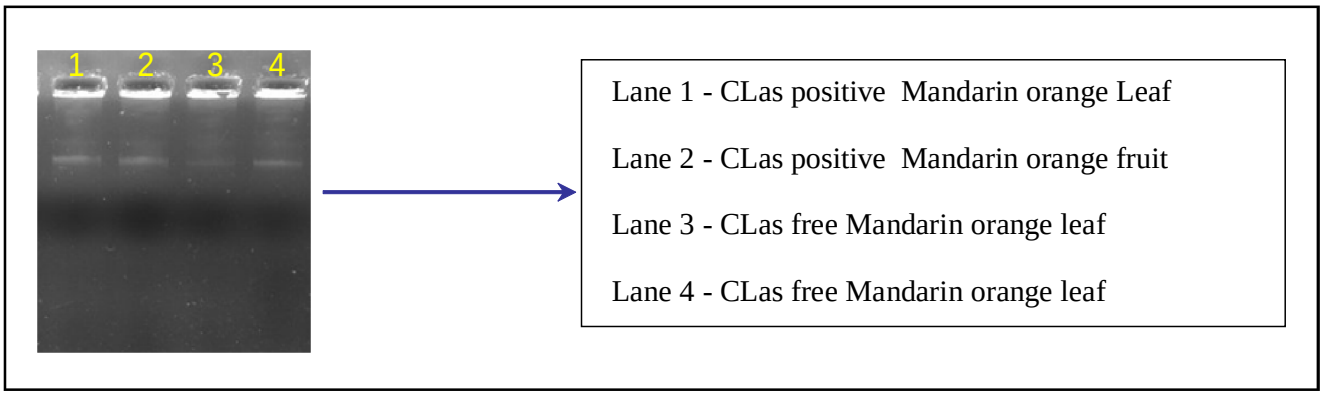
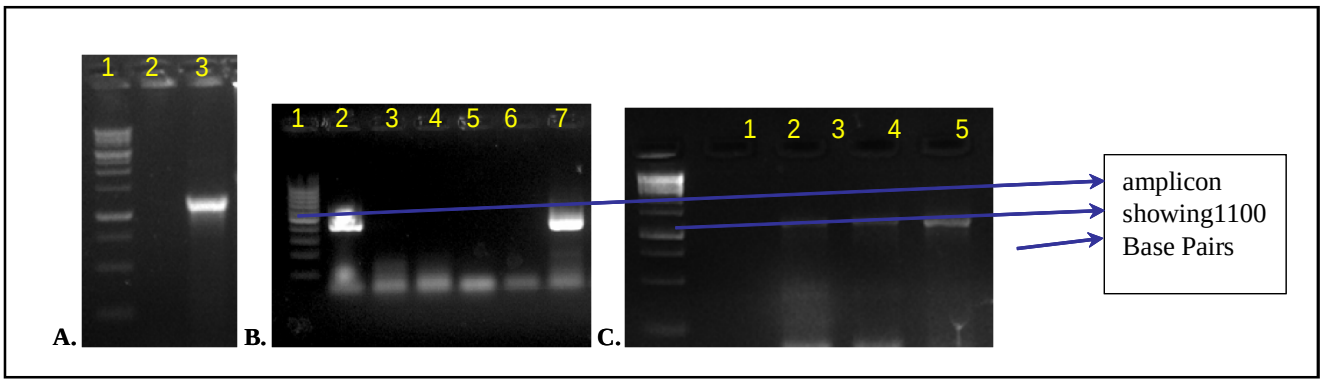


Figure 1: DNA isolation from both CLas positive and CLas free mandarin orange leaf, fruit.



A. Healthy and CLas positive mandarin orange fruit sample (Lane 1 and 2).
B. CLas positive and healthy mandarin orange leaf samples (lane 2,3,4,5,6) and positive control (Lane 7).
C. CLas positive leaf , CLas positive fruit and positive control (Lane 2,3 and 4).
*Lane 1 is 1 kb Ladder for all gsel pictures A, B and C.

Figure 2: Mandarin orange samples showing positive for *Candidatus* liberibacter asiaticus and healthy leaf samples showing negative.

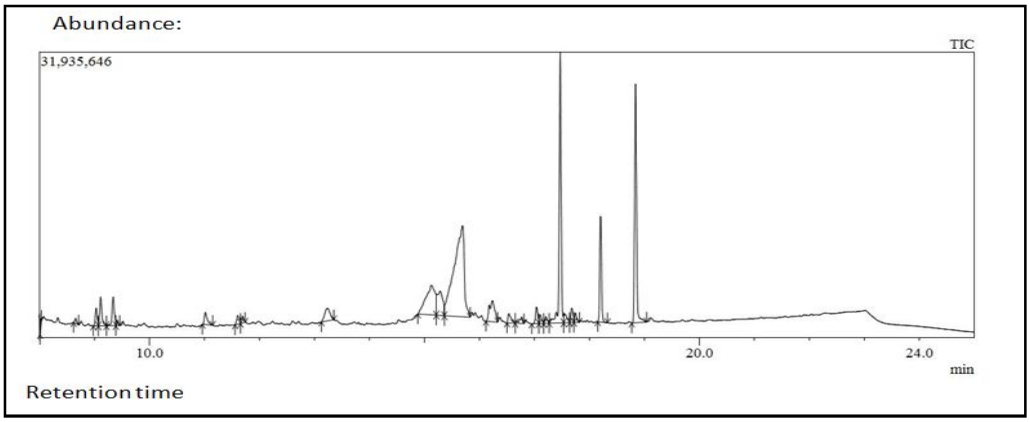


Figure 3: Total ion chromatogram obtained from healthy (CLas free) mandarin orange leaf sample.

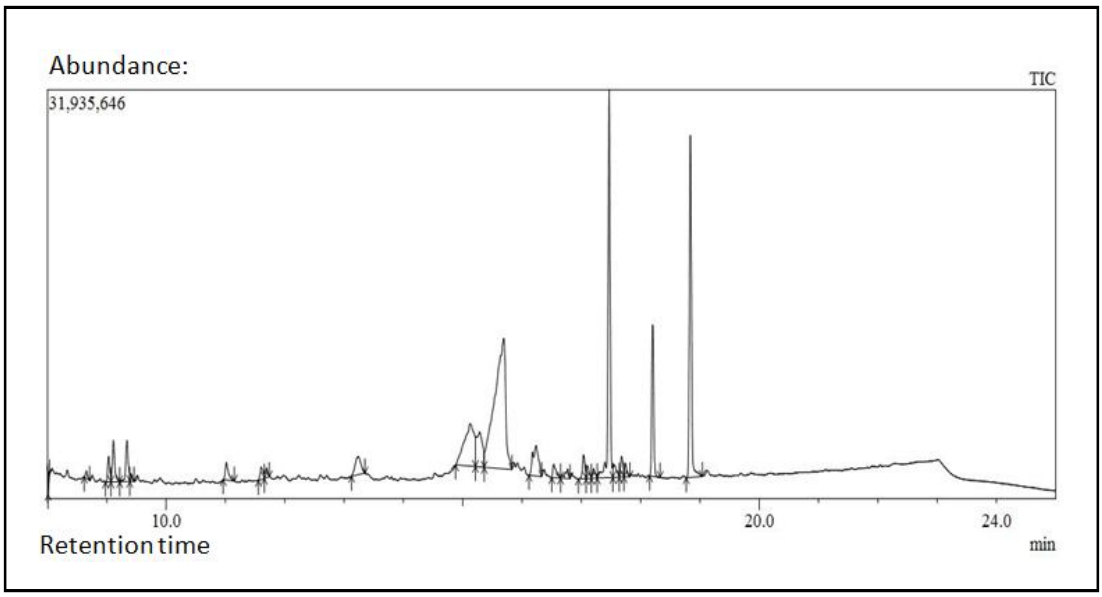


Figure 4: Total ion chromatogram obtained from CLas infected mandarin orangeleaf sample.

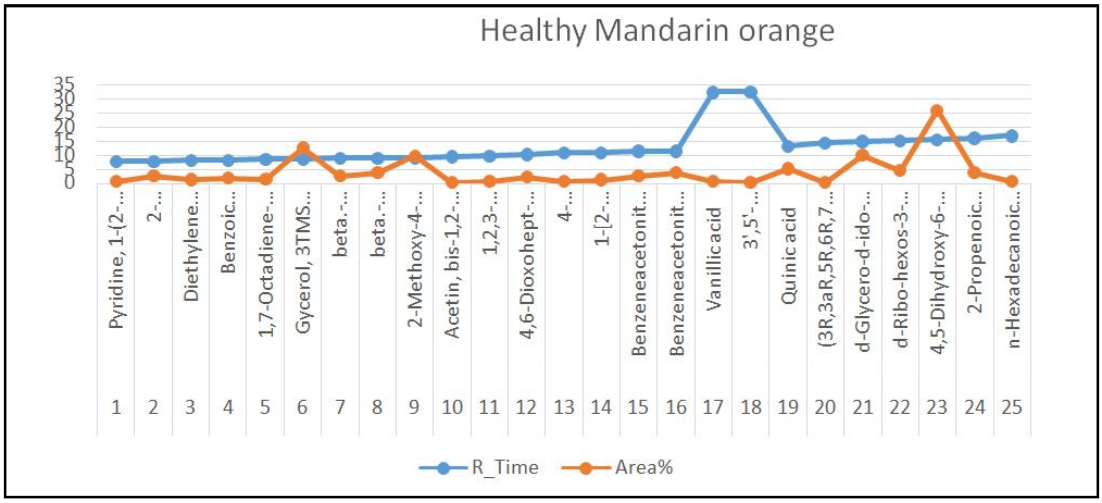


Figure 5: Graph showing the total compounds obtained through GC-MS analysis in healthy mandarin orange.

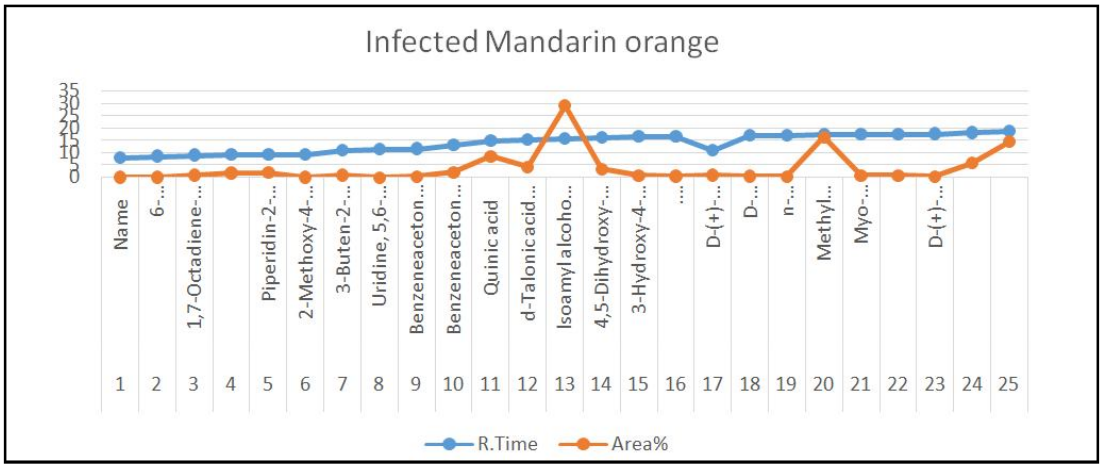


Figure 6: Graph showing the total compounds obtained through GC-MS analysis in CLas infected mandarin orange.

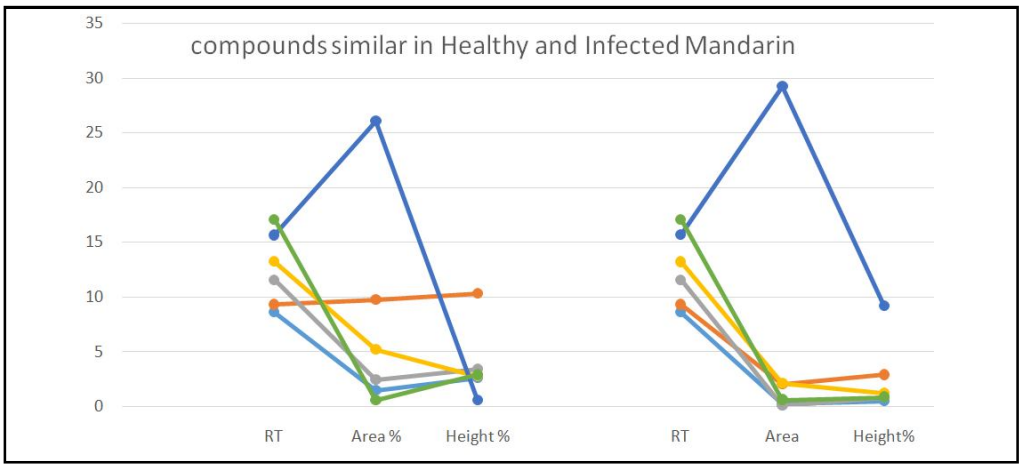


Figure 7: Line graph showing the compounds similar in healthy and infected mandarin orange and differentiated through area percentage.

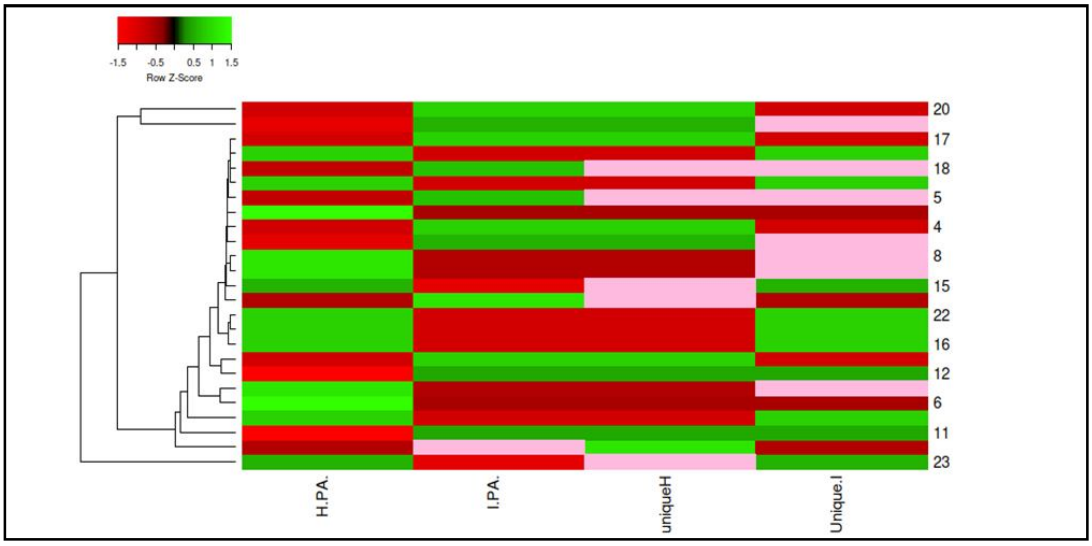
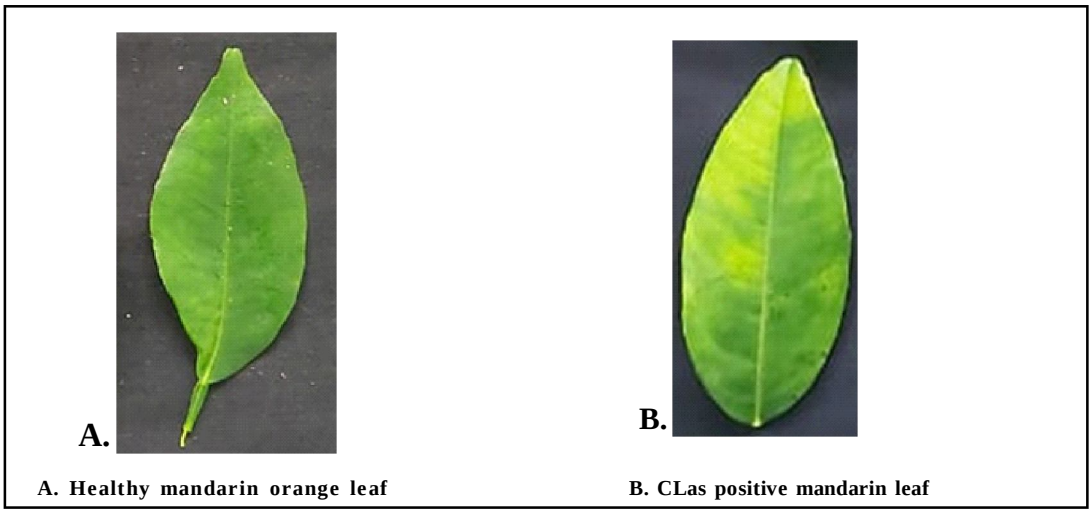


Figure 8: Heat map of gas chromatogram and mass spectrometry (Euclidean).



A. Healthy mandarin orange leaf **B. CLas positive mandarin leaf**

Figure 9: Leaf samples showing no symptom in healthy mandarin orange and blotchy, chlorotic appearance in CLas affected mandarin orange leaf.

4. Discussion

Numerous researchers have documented the biological and antimicrobial properties of the main monoterpene components found in citrus leaves as well as the release of monoterpene from injured or attacked leaf tissues. These monoterpene compounds have anti-insect properties and are involved in the induction of defense-related gene transcription in citrus. In perfume manufacturing, citronellol attracts pollinators and has insect repellent properties, especially for mosquitoes (Xie *et al*., 2018). Studies on bacteria such as *Pseudomonas citronellolis*, *P. aeruginosa* and *P. mendocina* strain (IBPse 105) that use citronellol, citronellal, and citronellyl acetate, myrcene as their carbon sources and helped to understand how monoterpenes are metabolised (Agwunobi *et al*., 2022; Tozoni *et al.*, 2010). Citronellal, one of the main terpenes in lemongrass cause reactive oxygen species to develop, which in turn causes mitochondrial depolarization, ATP depletion, and necrotic or apoptotic death in long-horned ticks (Agwunobi *et al*., 2021). Citronellol has been reported to have pharmacological actions such as antibacterial, antifungal, antispasmodic, hypotensive, vasorelaxant and anticonvulsant properties (de Sousa *et al*., 2006; Bastos *et al*., 2010; Santos *et al*., 2011).

Escherichia coli and *Proteus vulgaris* were thought to be resistant to chemical substances produced by the *Proteum hebetatum*, particularly p-Vinyl guaicol (Conrado *et al*., 2015). By boosting ATP hydrolysis or decreasing SDH activity, quinic acid may produce a large drop in intracellular ATP concentration, which would lower the DNA content of *S. aureus* cells (Bai *et al*., 2018). The longer delay in the reaction time when mice were exposed to a nociceptive stimulus during a hot plate test shows that C.T in all dosages has a central analgesic effect. The blocking activity of naloxone, a particular antagonist of morphinomimetic receptors, supported this central analgesic action (Mundey *et al*., 2000). These results imply like other monoterpenes like citronellal (Melo *et al*., 2010), a-terpineol and linalool (Peana *et al*., 2003), C.T.'s analgesic efficacy may involve the opioid system.

Damaged rough lemon leaves emitted volatile chemicals (citral, citronellal, and linalool), which dramatically reduced the formation of *alternaria* alternative hyphals and spores (Yamasaki *et al*., 2007). Additionally, *Xanthomonas citri* subsp. *citri* and *Penicillium digitatum* tolerance was generated in orange fruits *via* terpene down-regulation (Rodríguez *et al*., 2011). The majority of CLas-tolerant cultivars have high levels of aldehyde substances such asundecanal, neral, geranial and citronellal. These aldehydes might prevent CLas from moving around in the citrus phloem tissues. Aldehydes with antimicrobial properties include formaldehyde, glutaraldehydes, pelargon aldehydes, decanal, and benzaldehyde (Gao and chen, 2005; Dorman and Deans, 2000). In general, the antimicrobial compounds geranial and citronellal, the double bond is conjugated with the aldehyde group increasing their electronegativity (Dorman and Deans, 2000). These substances may disrupt biological processes involving electron transport as a result of their high electronegativity and they may interact with nucleic acids (Yamasaki *et al*., 2007). While citronellal was the only antibacterial agent effective against several microbes, geranial and neral had modest antibacterial action against a number of diseases. Increased b-citronellol and nerol synthesis in citrus fruit peels increased the fruit peels resilience to infections and insect attack. Similar findings were found in the current study as well β -citronellol is the most prevalent monoterpene, with a peak area percentage of 5.99% in the healthy leaves. Unsaturated monoterpene alcohols like β-citronellol have a light, energising scent and citrusy. Acyclic monoterpenoids like citronellol and dihydrogeraniol are found in nature, The most prevalent isomer is β -citronellol, which is present in citronella oils including *Cymbopogon nardus* (50%). The oils of roses (18-55%) and *Pelargonium geraniums* also contain β-citronellol. A significant amount of action against certain gram-positive bacteria was found in the essential oil of *Cymbopogon citratus* (lemongrass) (Boukhatem *et al*., 2014).The essential oil of *C. nardus* consists citronella has broad range of fungistatic activity against *Aspergillus*, *Chaetomium*, *Myrothecium*, *Pencillium*, and *Trichoderma* spp. (Khosravi *et al*., 2011; Delespaul *et al*., 2000).

The above studies provided adequate support for our findings and it was inferred from them that the predominance of beta citronellol in healthy acid lime leaves have antibacterial activity against gram-negative CLas in the phloem as well as insecticidal property such as mortality of psyllid vector that feeds on the phloem (*Diaphorina citri*). The use of beta citronellol alterations in the volatile profile act as an indication for CLas infection in citrus will greatly benefit additional validation and characterisation in this area of research. The volatiles found in our investigation, such as 2-methoxy-4-vinylphenol (Conrado *et al*., 2015), quinic acid (Bai *et al*., 2018), thymol methyl oxide (Feng *et al*., 2018) and geraniol (Kamatou and Viljoen, 2008) were also found to exhibit substantial antibacterial action against gram-negative bacteria in addition to beta citronellol. Sesquiterpenes such as b-sinensal, sinensal, farnesene, and germacrene were found in healthy acid lime leaves while they are entirely absent from leaves that have been infected with HLB.

5. Conclusion

The findings of the current investigation revealed that the healthy mandarin orange leaves contained more monoterpenes than the HLB infected leaves. These monoterpenes are signature candidates which act as potential biomarkers and plays animportant role in signalling pathway, thereby inducing plant defenses at low doses. The presence of citrus oil glands in the midribs, veins and phloem tissues of citrus leaves makes it possible for volatile chemicals to affect the CLas pathogen directly. Among these monoterpenes, beta citronellol was eluted to the highest concentration in the healthy mandarin orange leaves reported to have antibacterial activity against numerous gram negative bacteria and insecticidal activity against the phloem feeding insects in citrus. However, the various extrinsic factors such as the crop ecosystem, seasonal changes, other physical injuries, other diseases, infectionand varietal sensitivities, affect the volatile profile of every crop. From the early findings, monoterpenes and a combination of biomarkers from several metabolite groups can be used to detect HLB in non symptomatic citrus crop leaves. Additionally, this study provided a suggestion for adding beta citronellol to the volatile fraction of mandarin orange leaves monoterpenes for HLB detection. It would be excellent to create sensors for usage in the field based on variations in metabolite concentration.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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