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Huanglongbing disease induced changes in volatile profile of acid lime (*Citrus aurantifolia*. Swingle) trees

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Abstract
Huanglongbing (HLB) or Citrus greening (CG) disease is one of the destructive diseases causing severe crop decline in Citrus crops (Family: Rutaceae), which is caused by the pathogen, 'Candidatus *Liberibacter asiaticus'* (*CLas*). It is a systemic pathogen residing in the phloem tissues of Citrus crops. In India, HLB disease is emerging as a major disease in acid lime (*Citrus aurantifolia* Swingle.) crop. The pathogen and host interaction studies through metabolite profiling revealed the production of many terpene compounds, which are reported to have anti bacterial activity and induce signaling and defense responses in *Citrus* spp. The present study aiming at profiling the volatile composition of healthy and *CLas* infected *C. aurantifolia* leaf was carried out through Gas chromatography-Mass Spectrum (GC-MS). GC-MS chromatogram of the hexanal extract of *C. aurantifolia* leaves revealed the elution of two different pattern of volatiles in healthy and *CLas* infected acid lime leaf extract. Major volatiles with the remarkable peak area percentage observed in healthy acid lime leaves were, linalool (73.01%), ß-Sinensal (4.23%), Phytol (5.96%), α- Sinensal (1.15%), ß- Farnesene (1.61%), Germacrene B (1.51%), ß-elemene (1.29%). But distinct pattern of volatiles were observed in considerably higher amounts in *CLas* infected leaves. They were E- Citral (42.46%), ß- citral (25.58%), Geraniol/Lemonol (8.31%), Citronellal (3.84%), Phytol (2.56%) and Isocaryophyllene (2.22%). The outcome of the study revealed the major differences between these two profiles and also the dominance of linalool in healthy acid lime leaves. The pathogen is Gram negative and phloem residing one and transmitted by phloem feeding insect vector, Asian Citrus Psyllids (*Diaphorina citri*). Linalools were reported to have antibacterial activity against many Gram negatives including *Xanthomonas citri* pv. *citri* and insecticidal activity against phloem feeding insects. In Citrus plants, the oil glands are in the midribs and veins of the citrus leaves as well as in the phloem tissues, the direct effect of volatile compounds on the *CLas* pathogen and its vector is possible. An exhaustive cum successive volatile profiling studies in popular varieties, in different seasons and locations will be highly useful for using linalool changes in the volatile profile as an indicator for *CLas* infection in Citrus.

Keywords: acid lime, citrus greening disease, GC-MS analysis, volatile metabolites, terpenes

1. Introduction
Huanglongbing (HLB) or Citrus greening (CG) or is an emerging and one of the most destructive diseases infecting *Citrus* spp. (Family: *Rutaceae*). This disease is caused by the phloem limited non-culturable Gram negative bacteria called Candidatus *Liberibacter asiaticus* (*CLas*) and it is transmitted by the Asian Citrus Psyllid, *Diaphorina citri*. HLB disease causes symptoms like yellowing of the veins and adjacent tissues; followed by blotchy mottling of entire leaves, premature defoliation, die-back of twigs, decay of feeder rootlets and lateral roots, and decline in vigor, ultimately lead to the death of the entire plant.

Citrus plants are well known for its large volume of essential oils, and most of their components are monoterpene compounds (Bonaccors *et al*., 2012) [3]. Systemic infection of the pathogen, *CLas* in *Citrus* spp. leads to the changes in the characteristic metabolites composition of the essential oils. Terpenes are secondary metabolites and one of the dominant fractions among the *CLas* induced changes in the metabolite profile. Evidences are there for these compounds performing roles in signaling and inducing plant defenses. When these compounds are well characterized and designated with the *CLas* infection, this could be used as an inexpensive assay for field diagnosis of HLB disease.

Targeted metabolomics with the discriminatory metabolite analysis can be successfully employed for this (Cevallos- Cevallos *et al*., 2011) [10]. Similarly, GC-MS profiling of headspace metabolites in mangoes is utilised for differentiation of anthracnose and stem end rot diseases in mangoes (Moalemiyan *et al*., 2007) [13].

Though changes in concentration of no single compound may exclusively be attributed to HLB, the combined use of L-proline, β-elemene, trans-caryophyllene and α-humulene increased specificity (Rivas *et al.*, 2008) [14]. In addition, previous studies showed that essential oil components with a phenolic structure, such as thymol have the highest antibacterial activities. Presence of monoterpene alcohols, diterpene alcohols, thymol and its precursors at high levels in healthy *Citrus* spp. are the major contributing factors for their tolerance to *CLas* pathogen. It is hypothesized that identifying key metabolite specific for Citrus and HLB interaction and correlating them with the HLB resistance or tolerance may provide a lead to find out new HLB biomarkers. Hence this study was carried out with an aim to study the volatile profiles and to characterize the signatory metabolite for pre diagnosis of the HLB disease.

2. Materials and Methods

2.1 Sampling and experimental design

Six years old acid lime (*Citrus aurantifolia* Swingle) crop having the physiological maturity was chosen for the study. The authentic leaf samples of the variety, Balaji infected with HLB disease were collected during the month of July, 2021 from Puliyangudi village, Tenkasi district, Tamilnadu, India (Geo coordinates : 9.170N; 77.401E).

Each tree was divided into four quadrants (north, south, east and west) and fully expanded leaves remain attached with the twigs were collected from each of the quadrants and pooled. Healthy or asymptomatic leaves and those showing typical symptoms of HLB were collected and labeled properly (Batool *et al*., 2007) [2]. These were immediately placed with labels on ice in a cooler during transit to the laboratory, where they placed at 4˚C. These samples were subjected to polymerase chain reaction (PCR) using *CLas* specific primers for ascertaining *Clas* infection (Jagoueix *et al.*, 1996) [9].

2.2 Extraction of volatiles from acid lime leaves

Healthy and infected acid lime leaf (*C. aurantifolia* S.) samples (Fig 1) were categorized as PCR negative and positive based on PCR results. These leaf samples were ground to a fine powder separately and from each sample, one gram of the sample powder was transferred to two separate beakers containing 20 ml hexane and kept in sonicator for 30 minutes. The extracts were filtered using Whatman filter paper No. 1 and concentrated using rotary vacuum evaporator and diluted for further use (Craig *et al*., 1950) [4].

2.3 Volatile profiling of acid lime leaves

Volatile compounds of both healthy and infected acid lime leaf samples were determined with a Shimadzu Gas Chromatography unit equipped with a mass detector, Turbo mass gold containing a Elite-1 (100% Dimethyl Poly Siloxane), 30 m x 0.25 mm ID x 1 mM df. Conditions employed were; Carrier gas, helium (1ml/min); oven temperature programme 110°C (2 min) to 280 °C (9 min); injector temperature (250 °C); total GC time (45 min). The hexane extract was injected into the chromatograph in 1.0 ml aliquots. The major constituents were identified with the aid of a computer-driven algorithm and then by matching the mass spectrum of the analysis with the National Institute of Standards and Technology (NIST) library (Version. 2.0, year 2005). Software used for gas chromatography mass spectrometry (GC-MS) was Turbo mass – 5.1. This work was carried out at the Centre of Excellence for Innovation, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, India.

3. Results and Discussions

GC-MS chromatogram of the hexane extract of acid lime leaves showed the dominance of volatiles belonging to terpene compounds. Compounds with their peak area per cent, retention time, molecular formula, molecular weight, structure and its biological function were recorded. Volatiles were identified by mass spectra using NIST library.

The results of the study revealed the elution of 24 volatiles in n- Hexane extract of healthy acid lime leaves. The major compound detected in the chromatogram was linalool with a peak area of 73.01%, which is a monoterpene alcohol compound. The other compounds detected were Phytol (5.96%), sesquiterpene aldehydes *viz*., ß –sinensal (4.23%), α - Sinensal (1.15%), sesquiterpenes *viz*., ß – farnensene (1.61%), ß elemene (1.29%) and ß Germacrene (1.51%) (Table 1; Fig 2).

In HLB infected acid lime leaves, an entirely different pattern of volatiles comprising 25 compounds were observed. Major fraction of a compound detected in the chromatogram was E-citral with a peak area percentage of 42.46. Another dominant fraction was ß citral holding 25.58% peak area. Citral (lemonal), or 3,7-dimethyl-2,6-octadienal, is either a pair, or a mixture of monoterpene aldehyde with the molecular formula $C_{10}H_{16}O$. Monoterpene alcohol, Geraniol (Lemonol) was observed with 8.31% peak area, followed by a monoterpene aldehyde, Citronellal with a peak area percentage of 3.84. Phytol and isocaryophyllene were also found to be with a respective peak area of 2.56% and 2.22% (Table 2; Fig 3).

Volatile profile of the healthy acid lime leaves revealed the dominance of monoterpenes. Monoterpenol oxidative metabolism typically controls the formation of volatiles in flowers, fruits, and young leaves. The biological and antifungal activities of the major monoterpene components in the citrus leaves, induction of monoterpene release from leaf tissues by wounding or microbe attack, anti-insect activities, and induction of transcription of defense-related genes in citrus were reported by many workers. Paula *et al*., (2019) [5] reported that the presence of monoterpene alcohols, diterpene alcohols, thymol and its precursors at high levels in healthy acid lime leaves may contribute to its tolerance to *CLas* pathogen in Valencia oranges.

Comparable results were observed in the present study also; among the monoterpenes, linalools are the dominant ones with a peak area percentage of 73.01 in the healthy leaves. Linalools are unsaturated monoterpene alcohol and have light and refreshing odour with Citrusy smell. They are generally stored as glycosides and were reported to exhibit several biological activities such as antibacterial and antiplasmodial effects (Arctander, 1994) [1]. Vanzyl *et al.*, (2006) [16] also reported that linalools were accumulated abundantly in the plants belonging to the families like Lamiaceae, Lauraceae and Rutaceae (*Citrus* spp.). Linalools have been reported to have a strong bactericidal activity against Gram negatives and bacteriostatic against Gram positives (Vanzyl *et al.*, 2006) [16]. Besides linalool was also reported to express insecticidal property against the phloem-feeding insects (Gowan *et al*., 1995) [8]. More reasonable observation that justified the antibacterial activity of linalool was demonstrated by Shimada, 2021 [15]. He reported the indirect correlation between the linalool concentration and the titre of

Xanthomonas citri pv. *citri* in Ponkan mandarin trees.

These studies proposed sufficient substantiation to our findings and from them it could be presumed that the dominance of linalool faction in the healthy acid lime leaves might have expressed the anti-bacterial activity against the Gram negative *CLas* in the phloem and also might have killed the phloem feeding psyllid vector, (*Diaphorina citri*). Since the citrus oil glands are found in the midribs and veins of the citrus leaves as well as in the phloem tissues, the direct effect of volatile compounds on the *CLas* pathogen is possible. Further confirmation and characterisation in this line of work will be highly useful for using linalool changes in the volatile profile as an indicator for *CLas* infection in Citrus.

Other than linalool, the volatiles observed in our study *viz*., elemene (Fabri *et al*., 2019) [6] and α-terpinenol (Khadir *et al*., 2016) [12], thymol methyl oxide (Feng *et al*., 2018) [7] and geranial (Kamatou and Viljoen, 2008) [11] were also reported to have significant antibacterial activity against Gram negative bacteria. While observing the composition of sesquiterpenes, ß-Sinensal, α - Sinensal, β-Farnesene, β-Germacrene were recorded in healthy acid lime leaves, but they are completely absent in HLB infected leaves.

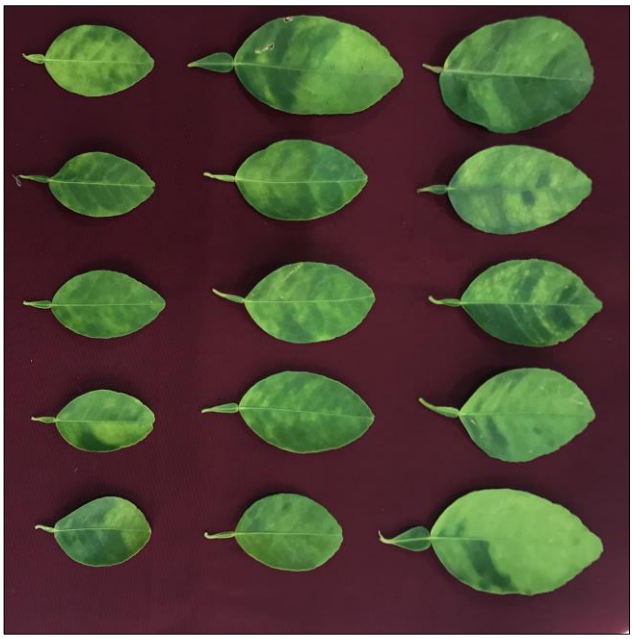


Fig 1: Acid lime leaves with HLB disease symptoms

Table 1: Volatile metabolites detected in n-hexane extract of healthy acid lime leaves

S. No.	Tentative Identity from NIST library	Volatile Compound Name	Retention time	% area	Molecular formula	MW
1.	Benzene	o-Xylene	5.019	0.32	$C_6H_4(CH_3)_2$	106.16
2.	Linalool	1,6-Octadien-3-ol, 3,7-dimethyl-	9.613	73.01	$C_{10}H_{18}O$	154.25
3.	α-terpinenol	3-Cyclohexene-1-methanol, α,α4-trimethyl-	12.368	1.15	$C_{10}H_{18}O$	154.25
4.	Monoterpene aldehydes Thymol methyl oxide	Benzene, 2-methoxy-4-methyl-1-(-1-methyethyl)	13.222	0.78	$C_{11}H_{16}O$	164.2441
5.	Geranial	2,6-Octadienal, 3,7-dimethyl-,(E)-	14.284	0.96	$C_{10}H_{16}O$	152.2334
6.	Surfynol 104	2,4,7,9-Tetreamethyl-5-decyn-4,7-diol	18.214	1.06	$C_{14}H_{26}O_2$	226.3550
7.	γ-Elemene	1-Methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-1-vinylcyclohexane), (1R-trans)-	18.854	1.29	$C_{15}H_{24}$	204.3511
8.	δ-Elemene	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	16.277	0.54	$C_{15}H_{24}$	204.3511
9.	Sesquiterpenes - β-(E)-Farnesene	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)-	19.336	1.61	$C_{15}H_{24}$	204.3511
10.	β-Germacrene	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	20.682	1.51	$C_{15}H_{24}$	204.3511
11.	Germacrene D-4-ol	(2E,4S,7E)-4-Isopropyl-1,7-dimethylcyclodeca-2,7-dienol	22.759	0.48	$C_{15}H_{26}O$	222.3663
12.	Hexadecamethyl-cyclooctasioxane	Cyclooctasiloxane,hexadecamethyl-	23.698	0.42	$C_{16}H_{48}O_8Si_8$	593.2
13.	Sesquiterpenes Aldehydeβ-Sinensal	2,6,11-Dodecatrienal, 2,6-dimethyl-10-methylene-	25.420	4.23	$C_{15}H_{22}O$	218.3346
14.	α-Sinensal	2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl-, (E,E,E)-	26.708	1.15	$C_{15}H_{22}O$	218.3346
15.	Octadecamethylcyclononasiloxane	Cyclononasiloxane, octadecamethyl-	27.225	0.63	$C_{18}H_{54}O_9Si_9$	667.3855
16.	Octasiloxane	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13	30.364	1.51	$C_{16}H_{48}O_7Si_8$	577.2
17.	Palmitic acid	Hexadecanoicacid,ethyl ester	31.818	0.47	$C_{18}H_{36}O_2$	284.4772
18.	Hexadecamethylcyclooctasiloxane	Cyclooctasiloxane,hexadecamethyl-	33.297	0.53	$C_{16}H_{48}O_8Si_8$	593.2315
19.	Phytol (stereoisomers)	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	34.429	5.96	$C_{20}H_{40}O$	296.5310
20.	Ethyl linoleate	Linoleic acid ethyl ester	35.642	0.54	$C_{20}H_{36}O_2$	308.4986
21.	Octadecamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	36.638	0.55	$C_{18}H_{54}O_9Si_9$	667.3855
22.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	39.736	0.54	$C_{18}H_{54}O_9Si_9$	667.3855
23.		Cyclononasiloxane, octadecamethyl-	42.486	0.53	$C_{18}H_{54}O_9Si_9$	667.3855
24.		Cyclononasiloxane, octadecamethyl-	44.932	0.55	$C_{18}H_{54}O_9Si_9$	667.3855

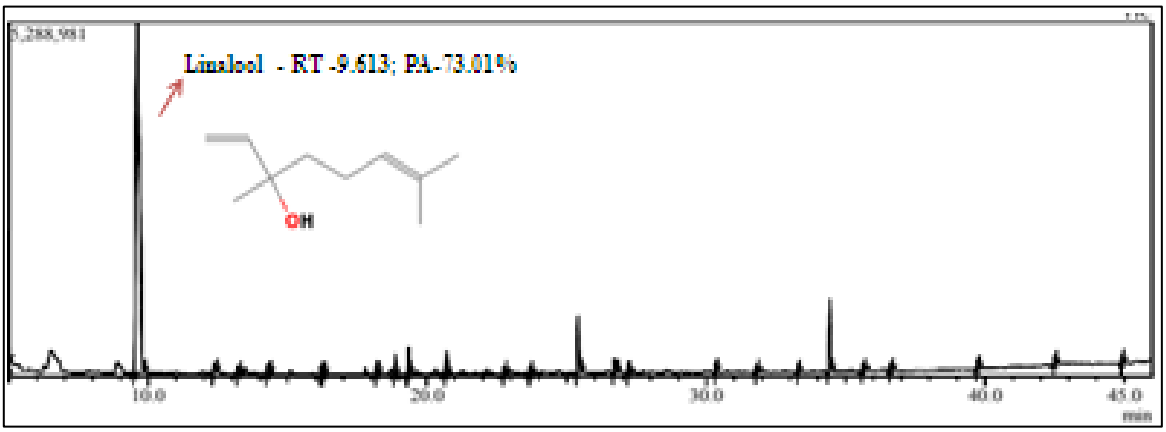


Fig 2: Typical Chromatogram of healthy acid lime leaves

Table 2: Volatile metabolites detected in n-hexane extract of HLB infected acid lime leaves

S. no.	Tentative Identity from NIST library	Volatile Compound Name	Retention time	Area%	Molecular formula	MW
1.	Benzene	o-Xylene	5.019	0.18	C_8H_{10}	106.1650
2.	Citronellal	6-Octenal, 3,7-dimethyl-,(R)-	10.996	3.84	$C_{10}H_{18}O$	154.2493
3.	Isogeranial	(E)-3,7-Dimethylocta-3,6-dienal; 3,6-Octadienal, 3,7-dimethyl-, (E)-	11.786	1.61	$C_{10}H_{16}O$	152.2334
4.	Nerol	2,6-Octadien-l-ol, 3,7-dimethyl-,(Z)-	13.117	1.32	$C_{10}H_{18}O$	154.2493
5.	Neral	2,6-Octadienal, 3,7-dimethyl-,(Z)-	13.500	25.58	$C_{10}H_{16}O$	152.2334
6.	Geraniol	2,6-Octadien-1-ol, 3,7-dimethyl-, (E)-	13.820	8.31	$C_{10}H_{18}O$	154.2493
7.	E-Citral (geranial)	2,6-Octadienal, 3,7-dimethyl-,(E)-	14.351	42.46	$C_{10}H_{16}O$	152.2334
8.	cis-Geranyl acetate	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (E)-	17.276	0.53	$C_{12}H_{20}O_2$	196.2860
9.	β-Elemene	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-	17.804	0.98	$C_{15}H_{24}$	204.3511
10.	(2E,3Z)-2-Ethylidene-6-methyl-3,5-heptadienal	3,5-Heptadienal, 2-ethylidiene-6-methyl-	18.520	0.64	$C_{10}H_{14}O$	150.2176
11.	Caryophyllene	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4E,9S*)]-	18.699	2.22	$C_{15}H_{24}$	204.3511
12.	γ-Elemene	1-Methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-1-vinylcyclohexane), (1R-trans)-	18.859	0.58	$C_{15}H_{24}$	204.3511
13.	(Z,E)-α-Farnesene	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (E,E)-	20.697	0.74	$C_{15}H_{24}$	204.3511
14.	β-Bisabolene	Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)-	20.866	0.69	$C_{15}H_{24}$	204.3511
15.	Caryophyllene oxide	5-Oxatricyclo[8.2.0.0(4,6)-]dodecane, 4,12,12-trimethyl-9-methylene-, [1R-(1R*,4R*,6R*,10S*)]-	22.935	0.41	$C_{15}H_{24}O$	220.3505
16.		Cyclononasiloxane, hexadecamethyl-	23.705	0.47	$C_{16}H_{48}O_8Si_8$	593.2315
17.	Isospathulenol	(1aR,7S,7aS,7bR)-1,1,4,7-Tetramethyl-1a,2,3,5,6,7,7a,7b-octahydro-1H-cyclopropa[e]azulen-7-ol	24.017	1.06	$C_{15}H_{24}O$	220.3505
18.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl	27.231	0.78	$C_{18}H_{54}O_9Si_9$	667.3855
19.		Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13	30.368	0.67	$C_{32}H_{72}O_{12}Si_8$	873.5909
20.		Cyclooctasiloxane,hexadecamethyl-	33.308	0.71	$C_{16}H_{48}O_8Si_8$	593.2315
21.	Phytol (stereoisomers)	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	34.437	2.56	$C_{20}H_{40}O$	296.5310
22.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	36.648	0.82	$C_{18}H_{54}O_9Si_9$	667.3855
23.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	39.744	0.73	$C_{18}H_{54}O_9Si_9$	667.3855
24.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	42.494	0.76	$C_{18}H_{54}O_9Si_9$	667.3855
25.	Octadeamethyl-cyclononasiloxane	Cyclononasiloxane, octadecamethyl-	44.937	1.34	$C_{18}H_{54}O_9Si_9$	667.3855

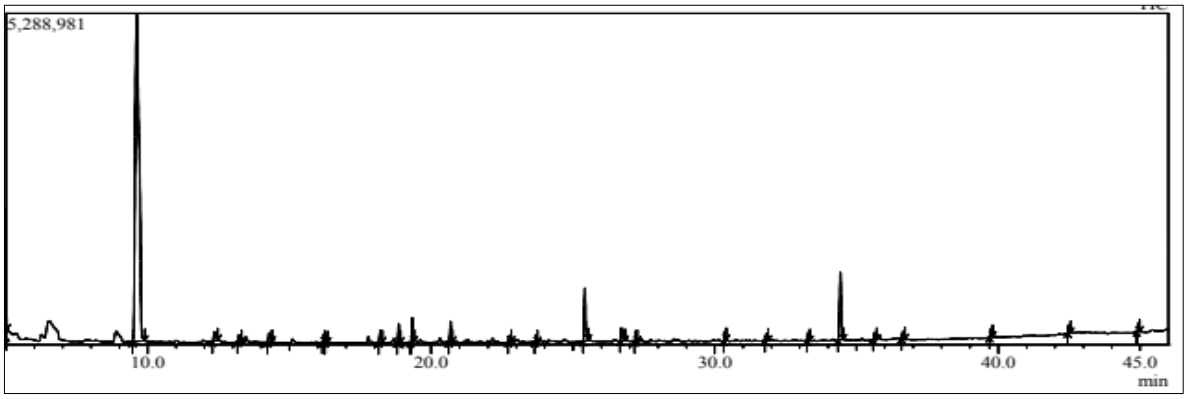
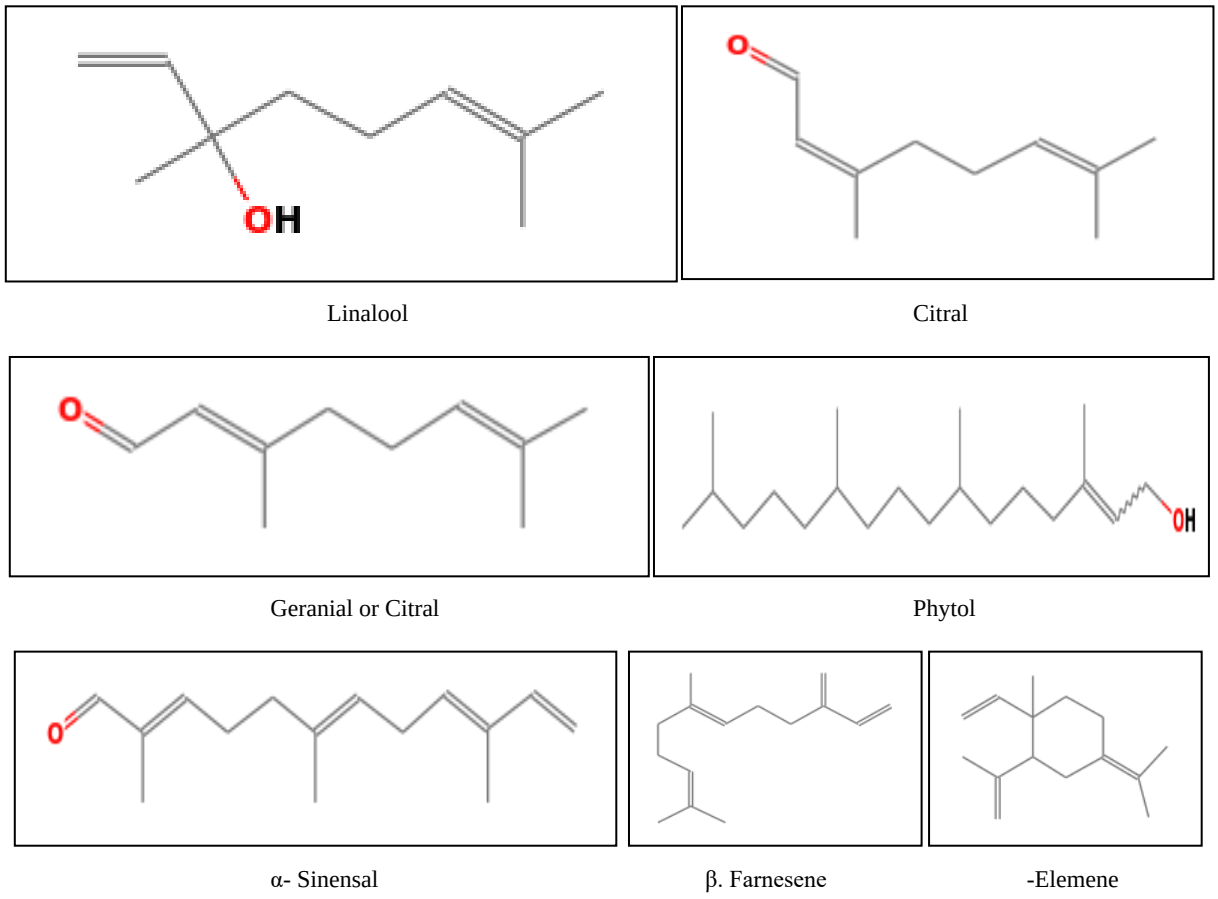


Fig 3: Typical Chromatogram of HLB infected acid lime leaves



Linalool Citral

Geranial or Citral Phytol

α- Sinensal β. Farnesene -Elemene

Fig 4: Chemical structure of important volatiles in acid lime leaves

4. Conclusion

In the present study, monoterpenes were found to be in a higher concentration in the healthy acid lime leaves than in HLB infected leaves. These monoterpenes are capable of performing roles in signaling and inducing plant defenses at low concentrations and they could be the best candidate for study as possible biomarkers. Since the citrus oil glands are found in the midribs and veins of the citrus leaves as well as in the phloem tissues, the direct effect of volatile compounds on the *CLas* pathogen is possible. Among these monoterpenes, linalool was eluted to a maximum concentration in the healthy acid lime leaves, which also has been reported to have antibacterial activity against many Gram negative bacteria and insecticidal activity against the phloem feeding insects in citrus. But the volatile profile of the any crop changes with the many external factors like crop ecosystem, seasonal changes, other physical injuries, infection by other pathogens and also varietal reactions too. Therefore, continuous exhaustive works have to be carried out under various weather conditions in various *Citrus* spp. and their varieties. Combination of biomarkers from different metabolite groups along with monoterpenes can be exploited for HLB detection in non-symptomatic leaves of Citrus crop. In addition, this study offered a lead to the inclusion of linalools under monoterpenes of volatile fraction of acid lime leaves for HLB detection.

5. Acknowledgement

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