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Tomato yellow leaf curl virus: Diagnosis and metabolites

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The existence of *Tomato yellow leaf curl virus* (TYLCV) was figured out in different locations in Al-Ahsaa of Saudi Arabia. Polymerase chain reaction (PCR) results of samples collected showed that TYLCV existed in all locations. Using AVcore and ACcore primers, begomoviruses family were detected in symptomatic tomato plants and by using TYv2664 and TYc138 (specific primers for the detection of TYLCV), the results proved that the samples were infected with TYLCV. The lipid-soluble fraction of healthy and infected tomato leaves extract was compared using gas chromatography techniques. A total of 46 compounds were identified in both healthy and virus-infected leaf tissues; among which 37 metabolites were common between both samples and increased or decreased in concentration due to the virus attack. Nevertheless, eight compounds were exclusively detected in the infected samples with only one compound consumed and thus recognized only in the healthy samples. The classifications and roles of the identified metabolites were discussed from the point of view of plant defense mechanisms or virus resistance against plant defense.

Key words: Tomato yellow leaf curl virus (TYLCV), begomoviruses, Polymerase chain reaction (PCR), gas chromatography.

INTRODUCTION

Tomato (*Solanum lycopersicum*, L.) is economically important in Saudi Arabia and is one of the most important vegetable crops in the world. It is considered as one of the most popular and widely grown vegetable crops worldwide with the area harvested in Saudi Arabia being 14,902 ha in 2016 (FAOSAT, 2016). The production of tomatoes in Saudi Arabia in 2016 was 503,217 tonnes with most of that production (60%) grown in greenhouses (FAOSTAT, 2016).

Begomoviruses have one (monopartite) or two (bipartite) genomic components, denominated DNA-A and DNA-B, and are transmitted in a persistent manner

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Figure 1. Healthy **(A)** and TYLCV-infected **(B)** tomato leaves. Picture on the left represent the healthy leaves and picture on the right represent infected leaves by TYLCV. The plant samples were taken from tomato fields and green houses in Al-Ahsaa, Eastern Province, Saudi Arabia.

by whiteflies of the species complex *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) to dicotyledonous plants (Fernandes et al., 2010). *Tomato yellow leaf curl virus* (TYLCV) is one of the most important harmful and invasive members of the genus *begomovirus* (family *Geminiviridae*), which is widespread over the world associated with tomato yellow leaf curl disease (Barboza et al., 2013). TYLCV is transmitted by whiteflies and can spread rapidly; it is also not transmitted through seed or by mechanical transmission. Severe symptoms such as leaf curling, stunting, and yellowing showed on TYLCV-infected tomato plants, which cause serious production loss in tomato cultivation (Kil et al., 2016; Papayiannis et al., 2010). In addition to tomato, other cultivated plants including pepper (*Capsicum* species), common bean (*Phaseolus vulgaris*), cucurbit (*Cucumis* species) and eustoma (*Eustoma grandiflora*) have been reported to be TYLCV hosts (Anfoka et al., 2009; Kil et al., 2016). Polymerase chain reaction (PCR) was applied to detect and establish provisional identity of begomoviruses through amplification of 575 bp fragment of the begomoviral coat protein gene (CP), referred to as the 'core' region of the CP gene (core CP). The core CP fragment contains conserved and unique regions, and was hypothesized to constitute a sequence useful for begomovirus classification (Brown et al., 2001).

Metabolomics is used nowadays as a high potential tool for understanding different metabolic changes in many biological systems and its applications have been recognized in the quality control validations and natural products research (Dai et al., 2010). However, the use of metabolomics in investigating interactions between different organisms is until now infrequent. For example, a metabolic profile for *Catharanthus roseus* leaves infected with phytoplasma has been determined (Choi et al., 2004) and the aromatic metabolite profiles of *Arabidopsis thaliana* infected by *Pythium sylvaticum* has been investigated (Bednarek et al., 2005).

To the knowledge of the authors, the metabolites that resulted from the interaction between the *Tomato yellow leaf curl virus* and its host the tomato plant (*S. lycopersicum*) has never been investigated. The main aim of this study was to explore the type of phytochemicals newly produced, increased or decreased in concentration during the attack of TYLCV on the tomato plant leaves. The study could ascribe to the better knowledge of the plant-virus chemical connection.

MATERIALS AND METHODS

Plant material

Healthy tomato (*S. lycopersicum*, family *Solanaceae)* leaves samples (HTL) were identified and collected from the local fields and greenhouses in Al-Ahsaa, Eastern province, Saudi Arabia. Meanwhile, TYLCV naturally-infected tomato leaves samples (ITL) showing the typical symptoms of TYLCV infection (severe stunting, yellowing, curling of leaves and chlorosis on leaves) were collected from same field greenhouses (Figure 1).

All plants were identified by experts and taxonomists in the College of Agriculture and Food Sciences, King Faisal University, Saudi Arabia with specimens deposited to the herbarium of the college.

Primers for begomoviruses and TYLCV

Two sets of primers were used in this study to identify the TYLCV infection (Table 1). The first set of primers were AVcore and ACcore used for the detection of begomoviruses and the second set of primers were TYv2664 and TYc138 used for the detection of TYLCV (Table 1).

Table 1. The sequences of the primers used to detect the Begomoviruses and TYLCV.

Viruses	Primers' Name	Nucleotide Sequences	Size (bp)	Reference
Begomoviruses	AVcore	5`- GCCHATRTAYAG RAAGCCNAGRAT -3`	575	Brown et al. (2001)
	ACcore	5`- GGRTTDGARGCATGHGTACANGCC -3`		
TYLCV	TYv2664	5`- ATTGACCAAGATTTTTACACTTATCCC -3`	316	Anfoka et al. (2005)
	TYc138	5`- AAGTGGGTCCCACATATTGCAAGAC -3`		

Extraction of total DNA from plant tissues

Total DNA was isolated from the infected tomato plants using DNeasy® Plant Mini Kit obtained from QIAGEN as manufacturer's instruction.

Polymerase chain reaction (PCR)

The extracted DNA was used as a template for PCR using set of primers as shown in Table 1. AVcore and ACcore primers were used as degenerate primers for begomoviruses group to amplify 575 bp while TYv2664 and TYc138 primers were used to amplify 316 bp of IR of TYLCV. PCR reactions were optimized for 25 µl and the final concentrations of reaction components were: 25 µM deoxynucleotide triphosphate (dNTPs), 2.5 µl of 10X PCR buffer, 2.5 mM $MgCl_2$, 5 units *Taq* DNA polymerase, 1 µl of 10 µM of each complementary and viral-sense primers and 3 µl of DNA were used as target templates. PCR cycle parameters for AVcore and AC core primers were as follows: one cycle at 94°C for 2 min; 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by one cycle at 72°C for 10 min. PCR cycle parameters for TYv2664 and TYc138 primers were as follows: one cycle at 94°C for 5 min; 30 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 56°C for 1 min, and 72°C for 10 min. Five microliters aliquots of PCR products were analyzed on 1% agarose gels in 0.5X TBE buffer.

Plant sample preparation for analysis

Three hundred grams of the healthy and TYLCV-infected tomato leaves were isolated directly from the field to make the HTL and ITL samples, respectively. The samples were immediately placed into liquid nitrogen for preservation and enzyme deactivation and then pulverized into a powdered form and kept in -20°C until further analysis.

Metabolites extraction

The powdered samples were extracted by n-hexane (HPLC-grade, Fisher Chemicals) using a Soxhlet apparatus for 3 h (20 cycles, each) according to Shah and Alagawadi (2011). The n-hexane extracts were evaporated under reduced pressure to yield different residues. Twelve plants were used to give six HTL and ITL samples.

Metabolites isolation and identification

The n-Hexane extracts were investigated using gas-chromatography-mass spectrometry (GC-MS) for qualitative analysis and gas chromatography-flame ionization detection (GC/FID) quantitative analysis. The GC conditions involved the use of Shimadzu-QP-2010 machine equipped with a capillary column (DB-5 ms 30 m × 0.25 mm I.D., 0.25 µm). The chromatograph was programmed for an initial temperature of 50°C for 2 min followed by a 5°C/min temperature ramp to 280°C. The final temperature was maintained for 4 min. Injector and detector temperatures are maintained at 250 and 280°C, respectively. The initial head pressure of the carrier gas (He) was 90 kPa and a split injection system (ratio 1:20) was used. In GC/MS, the capillary column was directly coupled to a quadruple mass spectrometer (Shimadzu model QP2010S), the ionization mode was electron impact (EI) and ionization energy was 70 eV.

Components identification and percentage area calculation

Different separated compounds were identified using Kovat's Retention indices (RI) calculated with respect to a set of co-injected homologous series of saturated hydrocarbon standards (C8 to C40, Sigma, UK). Compounds were identified by comparing their spectral data and RI with Wiley Registry of Mass Spectral Data 9th edition/NIST Mass Spectral Library (2011), and literature data (Adams, 2007). Some of the compounds were identified using authentic samples and those compounds are marked in Table 2. Calculations of peak percentage areas, based on FID response, are as follow:

Percent area of peak = (The FID peak area / the sum of all the FID peaks areas) × 100

Most of non-identified components are present as traces with relative abundances of less than 0.1%. The most important constituents identified in the n-hexane fractions analyzed are listed in Table 2. The percent area ratio was calculated for each component and displayed in Table 2. This ratio indicates that this component increased in concentration due to the virus attack (that is, the ratio will be more than 1) or decreased due to the attack (the ratio will be less than 1).

Statistical analysis

Six samples were used for both HTL and ITL (n=6), respectively and each sample was injected in triplicate. Quantitative values are expressed as mean ± standard error of mean of percentage areas and significance difference was determined using unpaired student-sample-t-test performed using SPSS statistical package version (SPSS for Windows, Version 11.5, SPSS Inc., Chicago, IL). $P<0.05$ was considered significant.

RESULTS

Detection of TYLCV

DNA of the expected sizes, 575 and 316 bp of

Table 2. Comparison between the n-hexan extract constituents of healthy tomato leaves (HTL) and infected tomato leaves (ITL) TYLCV. The major 46 compontes the n-hexan fraction were compared. The percentages of infected (ITL) to healthy (HTL) fraction of the same compound of both n-hexan fractions are in the ITL/HTL ratio column. The Quantitative values are expressed as mean ± SEM of six independent infected and healthy leaf samples (n=6).

S/N	Compound name	Rt (min)	RI	Area percentage		Significance difference*	ITL/HTL ratio#
				HTL	ITL		
1	Octane	2.444	801	0.208 ± 0.014	0.692 ± 0.036	Yes	3.327
2	1.3.5-Bisabolatrien-7-ol	21.151	1604	None	0.117 ± 0.014	Yes	Present in ITL only
3	E-Bisabol-11-ol	23.701	1666	None	0.057 ± 0.004	Yes	Present in ITL only
4	Palmitic acid**	24.268	1680	0.195 ± 0.008	0.116 ± 0.007	Yes	0.598
5	2Z.6E-Farnesol	26.012	1723	0.126 ± 0.024	0.162 ± 0.009	Yes	1.289
6	2E.6E-Farnesal	26.268	1729	0.048 ± 0.002	0.131 ± 0.018	Yes	2.711
7	E-β-Santalol	26.493	1735	0.309 ± 0.015	None	Yes	Present in HTL only
8	6R-7R-bisabolone	27.609	1762	0.256 ± 0.047	0.383 ± 0.025	Yes	1.499
9	β-Bisabolenal	27.771	1766	Trace	0.241 ± 0.029	Yes	Present in ITL only
10	2E,6E-Methyl farnesoate	28.38	1781	9.821 ± 0.424	8.379 ± 0.521	Yes	0.853
11	Z-β-Santalol acetate	30.042	1818	0.150 ± 0.024	0.095 ± 0.011	Yes	0.636
12	Phytol	30.953	1837	1.363 ± 0.124	1.645 ± 0.179	No	1.207
13	Linolenic acid methyl ester**	31.275	1843	1.546 ± 0.245	3.786 ± 0.168	Yes	2.449
14	Bisabolatrien-1-ol-4-one	31.377	1845	1.448 ± 0.256	1.643 ± 0.149	No	1.135
15	2E.6E-Farnesyl acetate	31.452	1847	0.925 ± 0.132	1.072 ± 0.087	No	1.159
16	Z.Z-Farnesyl acetone	31.649	1851	0.680 ± 0.055	1.176 ± 0.245	Yes	1.730
17	Stearic acid**	31.857	1855	1.582 ± 0.147	2.288 ± 0.196	Yes	1.446
18	E-β-Santalol acetate	32.243	1863	Trace	0.560 ± 0.084	Yes	Present in ITL only
19	5Z.9E-Farnesyl acetone	33.903	1897	Trace	0.708 ± 0.063	Yes	Present in ITL only
20	cis-9-Hexadecenal	34.775	1915	0.105 ± 0.065	0.095 ± 0.012	No	0.905
21	Isophytol	36.602	1952	None	0.504 ± 0.024	Yes	Present in ITL only
22	E-β-ionone	37.08	1962	0.763 ± 0.054	1.415 ± 0.092	Yes	1.855
23	Stearylaldehyde	37.518	1971	2.089 ± 0.045	0.546 ± 0.013	Yes	0.261
24	Kaur-15-ene	38.994	2001	0.165 ± 0.024	0.095 ± 0.007	Yes	0.578
25	Palmitaldehyde	39.657	2025	0.970 ± 0.057	1.567 ± 0.145	Yes	1.616
26	Kaurene	40.108	2041	0.696 ± 0.049	1.234 ± 0.098	Yes	1.772
27	6Z,10E-Pseudo phytol	40.292	2048	Trace	0.508 ± 0.041	Yes	Present in ITL only
28	6E,10E-Pseudo phytol	40.642	2060	None	0.690 ± 0.036	Yes	Present in ITL only
29	Humulene epoxide	41.524	2092	None	0.067 ± 0.084	Yes	Present in ITL only
30	Methyl linoleate	41.796	2102	0.681 ± 0.009	0.680 ± 0.007	No	0.999
31	Squalene	41.9	2105	0.706 ± 0.062	0.602 ± 0.041	Yes	0.852
32	Stearic acid methyl ester**	42.262	2118	0.757 ± 0.096	1.094 ± 0.112	Yes	1.444
33	linoleic acid**	42.683	2134	1.194 ± 0.145	0.999 ± 0.128	No	0.837
34	Oleic acid**	42.857	2140	Trace	0.486 ± 0.039	Yes	Present in ITL only
35	Abieta-(8(14).13(15)-diene	43.244	2154	6.020 ± 0.341	4.473 ± 0.418	Yes	0.743
36	Phytol acetate	44.951	2215	2.182 ± 0.156	1.808 ± 0.147	Yes	0.829
37	3-β-Stigmast-5-en-3-ol**	46.242	2261	2.104 ± 0.143	3.894 ± 0.221	Yes	1.850
38	Dehydro abietal	46.474	2270	0.844 ± 0.045	0.812 ± 0.061	No	0.962
39	β-Sitosterol**	47.138	2293	25.641 ± 1.597	20.256 ± 1.387	Yes	0.790
40	Abietal	47.459	2305	None	0.629 ± 0.025	Yes	Present in ITL only
41	Campesterol	48.646	2347	None	0.970 ± 0.087	Yes	Present in ITL only
42	Methyl dehydro abietate	48.939	2358	3.997 ± 0.367	3.171 ± 0.278	Yes	0.793
43	4-epi-Abietal	49.646	2383	3.213 ± 0.285	3.346 ± 0.391	No	1.041
44	Stigmast-4-en-3-one	51.642	2566	2.351 ± 0.135	2.282 ± 0.222	No	0.971
45	neo-Abietol	51.997	2605	None	0.566 ± 0.054	Yes	Present in ITL only
46	Lupeol**	52.918	2704	7.266 ± 0.521	6.604 ± 0.428	No	0.909

Table 2. Contd.

Total fraction percentage area	80.400	82.640	
Sesquiterpenes	24.701	25.945	
Fatty acids	9.118	11.656	
Sterols and triterpenes	38.068	34.607	
Abietic acid derivatives	10.938	11.154	
Bisaboline derivatives	1.703	2.440	
Phytol derivatives	3.544	5.155	

Rt, Retention time; RI, retention index. Trace: concentration less than 0.1%. *Significance difference is between H and I percentage area for each component, determined using unpaired student-sample-t-test (p<0.05). ** identified using standards compounds. #The ITL/HTL ratio indicate if this component increased in concentration due to TYLCV attack (implying the ratio is more than 1) or decreased due to the attack (implying the percentage is less than 1).

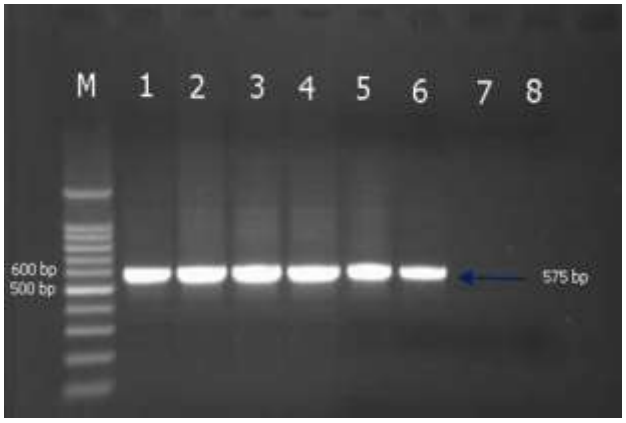

Figure 2. Agarose gel electrophoresis of PCR products using the degenerate primers AVcore/ACcore. M, 100 bp DNA ladder (Promega); from 1 to 6, six tomato samples showed symptoms of TYLCV infection; 7, healthy tomato sample; 8, negative control.

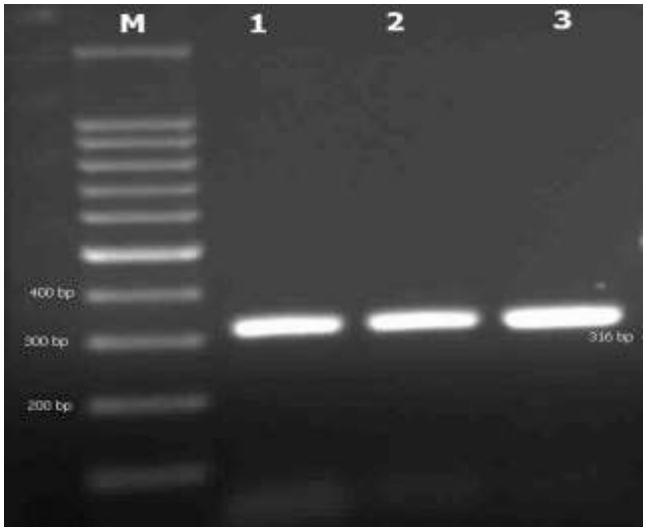

Figure 3. Agarose gel electrophoresis of PCR products using the primers TYv2664/TYc138. M, 100 bp DNA ladder (Promega); from 1 to 3, three tomato samples showed symptoms of TYLCV infection and gave positive reaction with degenerate primers AVcore/ACcore.

begomoviruses and TYLCV, respectively, were amplified from symptomatic tomato plants using the primer pairs AVcore/ACcore (Figure 2) and TYv2664/TYc138 (Figure 3), respectively.

Analysis of n-hexane fraction

This study was performed to compare the lipid-soluble metabolic pool of compound in healthy tomato leaves to that of the TYLCV-infected leaves, which allows the identification of the newly synthesized metabolites, or those, which differ in concentration because of the virus attack.

The gas chromatographic analysis of the n-hexane fraction (Figure 4 and Table 2) resulted in the separation of 84 components, 46 of which were identified, representing 80.40 and 82.64% of the total fraction contents of the HTL and ITL sample, respectively. Thirty-seven common compounds were identified between the two extracts; however eight compounds were produced uniquely in the infected tissue extracts and a compound was identified in the healthy samples only indicating its total consumption during the virus infection process. Similarly, five compounds were found in trace in the healthy tissues indicating that 14 compounds were more or less produced due to the virus infection (Table 2). The concentrations of the 12 compounds increased in the infected tissues in relation to the healthy ones. Nevertheless, ten common compounds decreased in concentration when the tissue was infected and both cases can be recognized from ITL/HTL ratio in Table 2. The change in concentration in ten common compounds was considered insignificant, and thus those compounds are considered to have no change in concentration due to the virus infection.

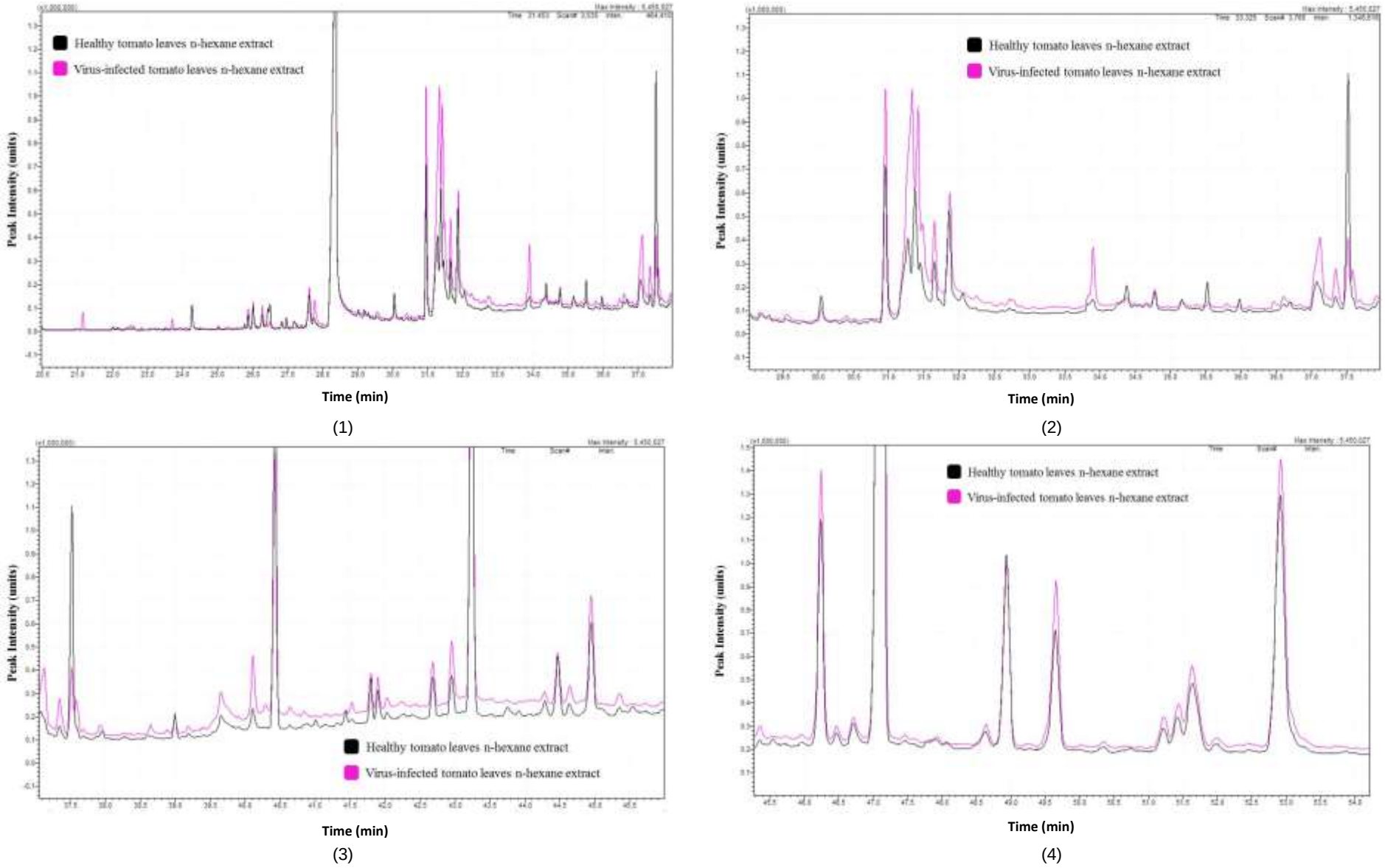

Figure 4. Aligned and expanded GC-MS chromatograms of the n-hexane fraction extracted from healthy Tomato leaves (HTL, lower in black) and TYLCV-infected leaves (ITL, upper in pink). To follow up the retention time on the chromatograms (from 1 to 55 min), follow the numbers from 1 to 4.

DISCUSSION

In the present study, the detection of begomoviruses in tomato was done and the results agreed with that of Alhudaib et al. (2014) and Rezk (2016) who used the degenerate primers of AVcore and ACcore to detect the begomoviruses in infected tomato samples in Saudi Arabia. Also, a leaf curl disease with symptoms typical of begomoviruses was observed in bean (*P. vulgaris*) at the Main Research Farm of the Indian Institute of Pulses Research, Kanpur, India (Kamaal et al., 2015) whereas Herrera-Vásquez et al. (2016) detected begomoviruses in commercial tomato plots using both production systems (open field and greenhouse) in Panama with different degenerate primers.

Just et al. (2014) stated that, imported tomato fruits infected with *Tomato yellow leaf curl virus* (TYLCV) were identified on the market in northern Europe using paper-based FTA Classic Cards (Whatman), PCR and partial DNA sequence analysis.

Impact of the metabolites pool changes due to the virus attack

Analysis of Table 2 discloses many classes of lipid-soluble components in the healthy and infected tissues of tomato leaves with different ratios. Sterols and triterpenes are the main class of compounds, which can be identified in the metabolites of both healthy and virus-infected leaves (Table 2). Although there was total decline in sterols and triterpenes concentration, 3-β-Stigmast-5-en-3-ol increased by 1.85 folds due to the virus infection. The concentration of β-Sitosterol represents nearly 25% of the total lipid-soluble fraction and it declined in the virus-infected leaves to reach 0.7 of its amount in healthy tissues. Accumulation of stigmasterol is a characteristic for plants during pathogens infection (Griebel and Zeier, 2010). Stigmasterol is chemically produced from β-sitosterol through C22 desaturation and this can explain the consumption of β-sitosterol and the increase in stigmasterol concentration due to the virus attack. Campesterol is produced uniquely in the virus-infected tomato leaves tissues. Campesterol are the precursor of steroidal phytohormones called brassinosteroids (Schaller, 2003), which are of vital role in plant defense mechanism against any pathogen attack (Choudhary et al., 2012). The increase in Campesterol concentration due to the virus attack might indicate an effect applied by the virus to weaken the plant defense through prevention of the production of brassinosteroids. On the other hand, a low ratio of Campesterol to sitosterol is needed for high plant cell membrane integrity and functionality (Schaeffer et al., 2001), though this ratio was affected by the production of Campesterol in the virus infected tissues which could lead to interruption and weakness of cell member, that is, the curling effect.

Sesquiterpenes are another class of compounds which are found in high ratio in the lipid soluble metabolic pool of tomato leaves. Although the total sesquiterpene compounds concentration insignificantly changed due to the virus infection, many individual components were produced exclusively or showed meaningful increase in concentration due to infection. Sesquiterpenes in tomato leaves metabolic pool can be divided into three main classes; Fernesols, bisabolines and abietic acid derivatives. Fernesols represent around 11.6% of the total lipid-soluble fraction of tomato leaves and although this whole ratio did not change due to the virus infection, many compounds were individually increased or decreased. The most abundant fernesol-type sequitepene in tomato leaf is methyl farnesoate, which represents 9.82% in healthy tissue, and this percentage decreased to 8.37 in the infected tissues. Fernesols are insect hormones (Nagaraju, 2007) and prevent fungal mycelia development with slight anti-fungal properties (Hornby et al., 2001). Bisabolene-type compound are another type of component that belong to the sesquiterpenes pool. Bisabolenes are recognized as sexual pheromones (Brézot et al., 1994; Lu and Teal, 2001) and thus they, together with Fernesols could be emitted by the plant to attract insects (War et al., 2012) as a way to resist the virus attack. Abietic acid derivatives is another class of sesquiterpenes, which is represented in the total lipid-soluble fraction tomato leaves extract by nearly 11%. This ratio did not change significantly between the infected and non-infected leave tissues. Abietic acid and its derivatives are diterpenes which are known for their role in plant defense mechanisms and are recognized for their tissue healing properties and pathogen trapping capabilities (Costa et al., 2016).

Phytols are acyclic diterpene alcohols, which decreased due to the virus attack on the plant. Although phytol and phytol acetate concentration decreased, isophytol was produced uniquely in the attacked tissues. The production of isophytol can explain the decrease in concentrations in phytol and its acetate. The role of isophytol as a production in the virus infected tissues is not clear and needs further investigation. Fatty acids have been identified in both the HTL and ITL samples with 9.11 and 11.6%, respectively. The main fatty acids found in both extracts was stearic acids and its derivative; stearylaldehyde and its methyl ester. The concentration of stearylaldehyde has dramatically decreased in ITL tissues to reach 0.2% of its original concentration in HTL. However, the concentration of stearic acid and its methyl ester has increased by 1.4% for both compounds due to the virus attack and this could explain the decline in stearylaldehyde concentration.

Conclusion

TYLCV has been identified in local area of Al-Ahsaa region, Eastern province of Saudi Arabia using means of

specific PCR primers. The fat-soluble metabolites resulting from the virus attack on the tomato plants has been revealed using means of GC/MS and quantified using GC/FID. Forty-six compounds were separated in both healthy and virus-infected leaf tissues, among which eight compounds were exclusively detected in the infected samples and only one compound was consumed and thus recognized only in the healthy samples.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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