RESPONSE OF *Ty*-RESISTANT TOMATO (*Solanum lycopersicum)* CULTIVARS TO BEGOMOVIRUSES AND THEIR GAS CHROMATOGRAPHY MASS SPECTROMETERY ANALYSIS

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ABSTRACT: Tomato (*Solanum lycopersicum)* production is affected by different diseases and *Tomato yellow leaf curl virus* (TYLCV) is the most prevalent and devastating begomovirus. *Ty*-resistant cultivars were developed by Asian Vegetable Research and Development Center (AVRDC) Taiwan, using different combinations of *Ty* genes to observe pyramiding effects on resistance against various Begomoviruses. Cultivars containing *Ty* genes were grown in glass house at 65% relative humidity, 16 hours Dark and 8 hours Light, at 26 ^{o}C. At the age of 5 to 6 leaves, plants were exposed to viruliferous whitefly and were kept for ~40 days. Symptoms were observed every 10 days post exposure. Among nine *Ty*-resistant and one native (Nagina) cultivars, three were resistant *i.e*., R1, R6 and R9, two mild to moderately resistant *i.e*., R2 and R10, three *i.e*., R7, R8 and R14 very mild resistant while one *Ty* cultivar *i.e*., R15 along with susceptible (Nagina) variety showed no resistance. Nagina (susceptible) and R6 (resistant) cultivars were analyzed by Gas Chromatography Mass Spectrometry (GCMS). Among *Ty* cultivars (*Ty*-2, *Ty*-3 and *Ty*-5) genes harbouring cultivars showed better resistance and GCMS analysis lead to identification of bioactive compounds associated with resistant (R6) cultivar.

Key words: *Solanum lycopersicum*, Begomoviruses, pyramiding, viruliferous whitefly and resistance.

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INTRODUCTION

Tomato (*Solanum lycopersicum*) is among the most important vegetables, grown all around the world (Bohs, 2005; Särkinen *et. al.*, 2013 andBakht and Khan, 2014). Approximately 163.4 million tons tomatoes are produced annually all around the world. In Pakistan tomato yield is about 574.0 thousand tons. Area under cultivation is 58 thousand hectares while its yield is 98 thousand hectogram per hector (Anonymous, 2013).

Among many devastating factors influencing tomato yield, whitefly transmitted geminiviruses (*Geminiviridae*) are prominent. *Geminiviridae* is classified into seven genera named as *Begomoviruses*, *Curtoviruses*, *Eragroviruses*, *Becurtoviruses*, *Masteri viruses*, *Turncurtoviruses* and *Topocuviruses*, on the basis of host range, insect vector and genome organization (Adams *et. al.*, 2013). Begomoviruses consists of monopartite (only DNA-A) or bipartite genome having two **(**DNA-A and DNA-B**)** genomic components.

Begomoviruses pose serious threat for tomato production by causing tomato yellow leaf curl disease (TYLCD) and tomato leaf curl disease (ToLCD) (Prasanna *et. al.*, 2015a). About 70 begomoviruses naturally infecting tomato have been identified by (Tsai *et. al.*, 2011). Asian vegetable research and development center (AVRDC) virologists identified tomato leaf curl disease in1981, for the first time in Taiwan the causative agent is a monopartite *Tomato leaf curl Taiwan virus* (ToLCTWV), latter on a bipartite begomo virus *Tomato yellow leaf curl Thailand virus* (TYLCTHV) became equally prevalent which is more devastating (Tsai *et. al.*, 2011).

To control this disease merely controlling whitefly population is labor intensive, expensive and sometimes proves unsuccessful. Accordingly breeding resistance, against begomoviruses is imperative goal of tomato breeding programs to control ToLCD. Wild tomato species being resistant against begomoviruses contain *Ty* (referred *Ty* for TYLCV resistance) genes (Prasanna *et. al.*, 2015b). Six *Ty* (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-3a*, *ty-5* and *ty-6*) resistance genes, up till now have been derived from wild (*Solanum chilense, S. habrochaites, S. peruvianum, S. pimpinellifolium*) tomato species (Prasanna *et. al.*, 2015a and Ji *et. al.*, 2007). Use of resistant cultivars, provide a good solution for the disease management (Caro *et. al.*, 2015 and Prasanna *et. al.*, 2015b).

However these *Ty* genes identified may not be equally efficient against all strains or species of begomoviruses. Thus, in order to develop tomato lines suitable for various locations and to acquire long lasting resistance, a strategy is adapted to pyramid various *Ty*

genes in the same tomato variety. AVRDC have produced cultivars, combining *Ty-1, Ty-2, Ty-3/Ty-3a, ty-5*and *'ty6'* genes (Hanson *et. al*., 2011). So single or combinations of *Ty* genes are required to be assessed against various begomoviruses species/ strains, in different areas to determine single or pyramidal genes affectivity (de Resende *et. al.*, 2009).

Plants continuously are exposed to a wide range of pathogenic organisms, below and above the ground, to control pathogens an extensive use of chemical pesticides lead to health and environmental hazards. Plants have evolved several means to recognize and defend against infection (Buhtz *et. al.*, 2015). So exploitation of natural plant products and metabolites for antimicrobial activity, is required to treat infectious diseases in plants and human (Das *et. al.*, 2010). Photochemicals include primary and secondary metabolites involved in plant defense which belongs to three main classes *i.e.*, phenolics, terpenoids and alkaloids (Doshi *et. al.*, 2015). Plants produce, about 50,000 secondary metabolites with known structures including terpenoids, phenylpropanoids, alkaloids and other compounds (De Luca and St Pierre, 2000).

Present study was conducted to determine efficiency of *Ty*-resistant tomato lines using viruliferous whiteflies and GCMS analysis of a resistant and highly susceptible cultivar was carried out.

MATERIALS AND METHODS

Plants growth conditions: *Ty* resistant seeds obtained from AVRDC Taiwan and one native susceptible (Nagina) tomato variety (n=15 seeds each), were sown in earthen pots of about 10 inch diameter, filled with silt, clay, sand and compost in almost equal proportion. These pots were placed in relative dark (16 hrs.), light (8 hrs.) with (65%) humidity at 26 °C. Plants were watered daily and with Hoagland soln. once in a week. Hoagland soln consists of (1.5 mM $Ca(NO_3)_2.4H_2O$, 0.75 mM $MgSO_4.7H_2O$, 1.25 mM KNO, 0.5 mM KH_2PO_4, micronutrients [15µM $MnCl_2.4H_2O$, 50 µM H_3BO_3, 2.0 µM $ZnSO_4.7H_2O$, 1.5 µM $CuSO_4$. $5H_2O$, 0.5µM $Na_2MoO_4.2H_2O$] and Fe-EDTA [1mM KOH, 30 µM $FeSO_4.7H_2O$, 30 µM EDTA]).

Seedlings after appropriate growth and height (15 days post sowing) were transferred individually into new plastic pots with 5 inch diameter having compost, clay, sand and silt.

Exposure through viruliferous whiteflies: Viruliferous whiteflies were collected from infected cotton and tomato fields adjacent to National Institute for Biotechnology and Genetic Engineering (NIBGE), and were allowed to infect seedlings. In this area, viruliferous whiteflies are carrier of *Cotton leaf curl Multan virus*, *Cotton leaf curl Khokhran virus*, (Burewala strain), *Tomato leaf curl New Delhi virus* and *Tomato leaf curl virus,* which were inferred on the basis of symptoms similarity. Tomato seedlings at the age of 4 to 5 leaves were used for exposure under same conditions in glass house.

The plants were regularly observed for virus symptoms after an interval of 10 days. Symptoms showing plants were observed carefully and change in infection level was noted. Phenotypically the plants were divided in five categories based on symptoms severity, from non-symptomatic (0), to mild symptomatic (1), medium symptomatic (2), severe symptomatic (3) and very severe symptomatic plants (4).

Symptoms severity bar graph was constructed by taking mean S.D and were compared by ANOVA.

Mean S.D and ANOVA values for symptoms severity among cultivars

Symptoms severity	NAG	R1	R2	R6	R7	R8	R9	R10	R14	R15
Mean	2.83	-	1.33	-	1.67	2.00	-	0.83	2.00	2.50
Rank by Mean	1	8	5	7	4	3	4	3	2	1
S.D	0.98	-	0.82	-	0.52	0.63	-	0.41	0.89	0.55
Mean- S.D	1.85	-	0.52	-	1.15	1.37	-	0.43	1.11	1.95
Mean+ S.D	3.82	-	2.15	-	2.18	2.63	-	1.24	2.89	3.05

Groups	Sum	Average	Variance
NAG	17.00	2.83	0.97
R1	-	-	-
R2	8.00	1.33	0.67
R6	-	-	-
R7	10.00	1.67	0.27
R8	12.00	2.00	0.40
R9	-	-	-
R10	5.00	0.83	0.17

R14		12.00		2.00		0.80
R15		15.00		2.50		0.3
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	61.15	9	6.79	19.05	0.00	2.0734
Within Groups	17.83	50	0.36			
Total	78.983	59				

Similarly bar graph for gradual increase in symptomatic plant number was constructed by using percentage values calculated by measuring mean S.D and were compared by ANOVA.

Mean S.D and ANOVA values for percentage of infected plants

Percentage of Infected plants		R7	R8	R9	R10	R14	R15
	Mean S.D	42%	54%	0%	33%	37%	54%
		50%	53%	0%	38%	44%	53%
	Mean S.D-	- 0.08	0.01	-	- 0.05	- 0.06	0.01
	Mean S.D+	0.92	1.08	-	0.72	0.81	1.08
Groups			**Sum**			**Average**	
10 Days			-			-	
20 Days			0.83			0.08	
30 Days			5.83			0.58	
40 Days			6.50			0.65	

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	3.363	3	1.12	11.02	0.00	2.87
Within Groups	3.664	36	0.10			
Total	7.027	39				

Polymerase Chain Reaction: Three plant samples were harvested to evaluate systemic infection via PCR, according to symptoms severity level in every cultivar, at about 40 days after exposure. DNA was extracted from selected plant samples by CTAB method (Doyle and Doyle 1990). Conventional PCR was performed by using ND-A qPCR F/ ND-A qPCR R primer pairs designed for begomoviruses which amplify fragment of 200 base pairs, sequence shown below.

ND-A qPCR F: 5′GTCGAAGCGACCAGCAGATAT3′

ND-A qPCR R: 5′GGAACATCTGGACTTCTGTAC3′

PCR procedure: DNA was amplified in PCR tubes of 0.5 ml capacity containing genomic DNA (50-100 ng), 10X *Taq* Polymerase buffer 5µl (Thermo), 5µl dNTPs (2 mM), 1 µl (0.5 µM) of forward and reverse primers, MgCl2 3µl (1.5 mM) and 1 µl (1.25 units) *Taq* DNA polymerase (Thermo). Reaction was completed by using specific PCR profile. Preheating of genomic DNA was done at 94^{o}C (5 minutes) followed by denaturation, annealing and extension, at 94^{o}C (30 seconds), 58-60^{o}C (30 seconds), 72^{o}C (45 seconds) respectively. Latter this 72^{o}C temperature was kept for 10 minutes, PCR reaction was taken out from PCR machine and run on 1.5% agarose gel.

Gas chromatography mass spectrometry: Plant samples were shade dried for about 2 to 3 weeks, weighted, ground fine and 1 gram sample was soaked in 10ml of n-hexane in flasks (250mL) which were shaked vigorously to let all the materials get soaked in the solvent. Extracts were filtered by using Whatman filter paper (11µm). About 2 to 3 mL extracts obtained were stored at 4^{o}C for further use by GCMS.

By following protocol described by (Ibrahim *et. al.*, 2011) with modifications, bioactive compounds were analyzed using GC-MS (QP2010 plus Shimadzu, Japan) and DB-5 capillary column (30mx0.25mm, film thickness 0.25µm J&W scientific, Folsom C.A). Helium was used as carrier gas, oven temperature was kept at 50^{o}C for 5minutes, latter programmed from 50-100 at 10^{o}C/min and maintained at the final temperature for 5minutes. Injector and detector temperature were kept 200 and 250^{o}C respectively. Ionization voltage was 70eV with mass scan range of m/z 55-950. For unknown compounds identification, mass spectrum comparison of the constituents with National Institute Standard and Technology library were taken (NIST-2006). Then compounds molecular formulas, retention time (RT), molecular weight and abundance percentage were tabulated.

RESULTS AND DISCUSSION

Ty resistant and susceptible (Nagina) cultivars at the stage of 4-5 leaves were exposed to viruliferous whitefly. Data was collected for all plants of resistant and

susceptible cultivars after 10, 20, 30 and 40 days of exposure. Data collected at 10 days exposure indicated that all plants of *Ty*-resistant cultivars along with native (Nagina) variety were found non-symptomatic and phenotypically normal. At 20 days exposure plants were also observed and data collected indicated very less number of plants found symptomatic which exhibited mild phenotypic changes like mild leaf vein thickening and relatively small plant size than phenotypically normal plants. In order to observe enhancement of symptoms severity and increase in number of symptomatic plants with the passage of time, data was collected after 30 days and 40 days of exposure. Data clearly represented increase in symptoms severity with increasing time (Fig-1).

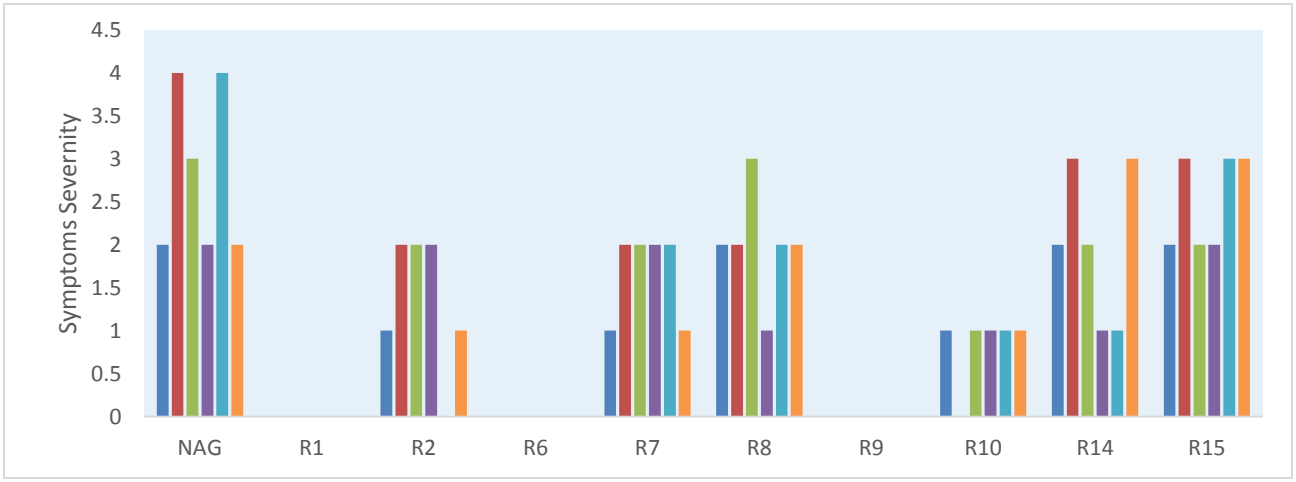

Fig-1: Symptoms severity shown by *Ty* cultivars and Nagina at 10, 20, 30 and 40 days.

Similarly, infected plants numbers were also compared with increase in time, which increased gradually till 40 dpi.

Along with symptoms severity, data clearly exhibited increase in numbers of infected plants, after exposure. All plants of R1, R6 and R9 cultivars were phenotypically healthy till 40 Dpi. While R2, R7, R8, R15 and Nagina showed 100% diseased plants, but R10 showed 66.66 and R14 showed 83.33% infected plants (Fig-2).

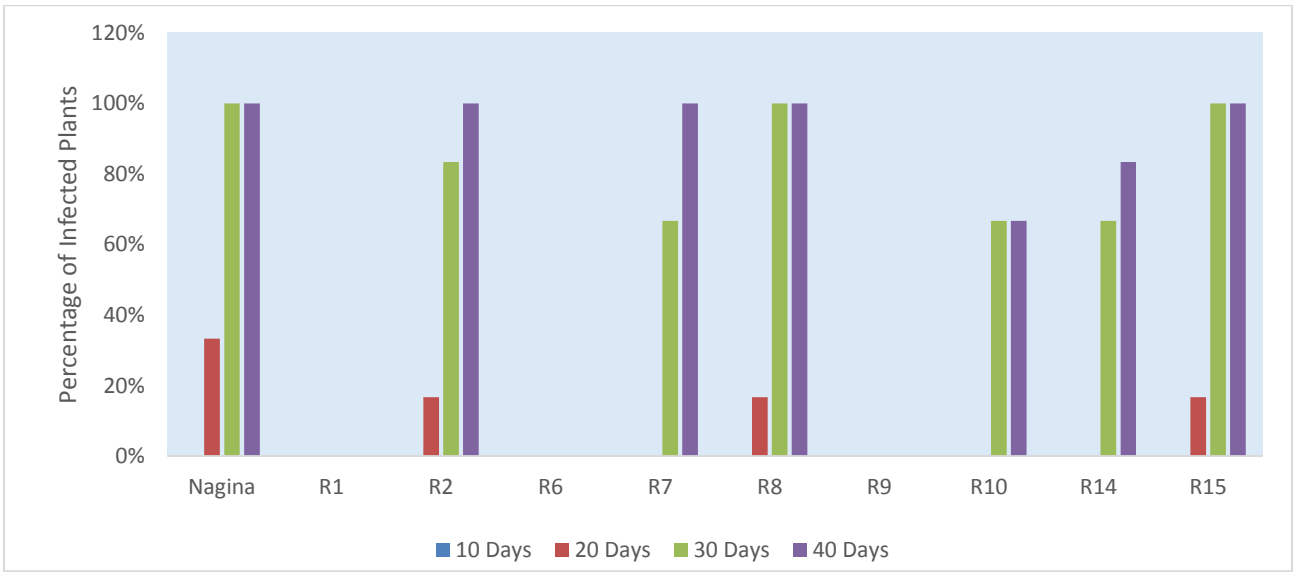

Fig-2: Gradual increase in number of symptomatic plants after whitefly exposure.

After 40 days of exposure, harvested plant samples (three for each cultivar) were used to extract DNA to perform PCR to confirm the presence of begomoviruses in the samples. All samples were found PCR positive (Fig-3).

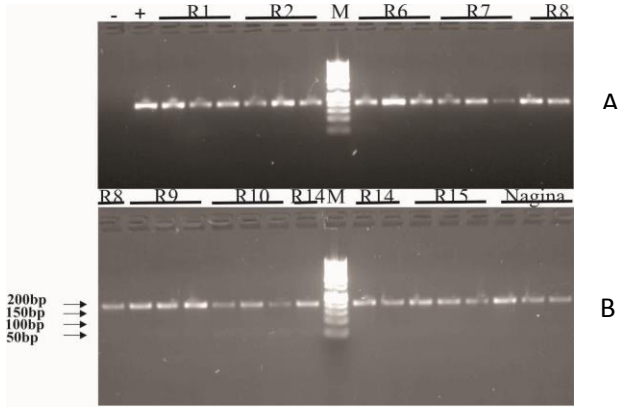

Fig-3: PCR amplified product run in comb (A) labeled as + and - indicated, positive and negative control respectively. Wells were marked as R1, R2, R6, R7, R8 run in comb were (A) R9, R10, R14, R15 and Nagina in comb (B). M represents DNA marker to compare fragment length indicated all infected samples were positive and give amplification of 200bp.

Among *Ty* cultivars used in study, all plants of R1, R6 and R9 cultivar were found symptomless during the whole experimental period. As pyramiding was based on multiple genes stacking, this ultimately led to the simultaneous expression of many genes, in a variety to produce reliable resistance expression (Joshi and Nayak, 2010). These three *Ty*-resistant cultivars were homozygous resistant for the *Ty-2* and *Ty-3* genes, while homozygous susceptible for the *Ty-1/Ty-3*and *Ty-5* genes (Table-1).Tomato hybrids homozygous for *Ty-1* and *Ty-2* were previously evaluated in a study against monopartite *Tomato yellow leaf curl virus* (TYLCV), *Honeysuckle yellow vein mosaic virus* (HYVMV) and *Tobacco leaf curl Japan virus* (TbLCJV) (Shahid *et. al.*, 2013). Resistance response indicated that *Ty-1* hybrids were resistant to moderately resistant against *Honeysuckle yellow vein mosaic virus*, as a large plant number found neither symptoms free nor virus free and were found PCR positive for virus (Shahid *et. al.*, 2013). In comparison *Ty-2* hybrids were found highly tolerant against three begomoviruses species (Shahid *et. al.*, 2013). In s study *Ty-1* genes showed mild symptoms development, but *Ty-2* carrying tomato plants showed complete resistance against TYLCV but were not found completely resistant against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and (Barbieri *et. al.*, 2010).

Table-1. *Ty* resistant cultivars with distribution codes and various *Ty* genes combination

		***Ty* genes combination**						
Cultivars Name	**Distribution code**	**Ty-1/Ty-3**	**Ty-2**	**Ty-3**	**Ty-5**	**Symptoms severity**	**PCR results**	**Symptoms at ~40 Dpi $**
R1	AVTO1008	S	R	R		No Symptoms	Positive	NS
R2	AVTO1010	S	R	R		Moderate Symptoms	Positive	LC, LR, LT
R6	AVTO1005	-	R	R	S	No Symptoms	Positive	NS
R7	AVTO1132	S	R	R	S	Severe Symptoms	Positive	LT, VT, EN, LR
R8	AVTO0922	R	R	S	S	Severe Symptoms	Positive	LT, LC, LR, VT, EN
R9	AVTO1130	S	R	3a	S	No Symptoms	Positive	NS
R10	AVTO1122	S	R	S	R	Mild Symptoms	Positive	VT, LT
R14	AVTO0301	S	R	S	S	Severe Symptoms	Positive	LT, VT, LY, GS, LR
R15	AVTO1080	S	S	S	S	Very Severe Symptoms	Positive	LT, LC, CL, GS, LR, VT
Nagina						Very Severe Symptoms	Positive	LY, LC, GS, LR, VT

Ty-1, *Ty*-2, *Ty*-3 and *Ty*-5 genes showed resistance to tomato yellow leaf curl disease, R Homozygous for resistance, S Homozygous for susceptibility, *Ty*1/*Ty*-3 both were allelic, -indicated uncertainty about *Ty*1 allele,3a indicated presence of the *Ty*-3a allele at the *Ty*-3 locus, $ Symptoms were observed in inoculated plants were denoted as leaf Thickening (LT), leaf curling (LC), leaf yellowing (LY), growth stunting (GS), leaf rolling (LR), Enation (EN), leaf yellowing (LY), vein thickening (VT), crumpled leaves (CL) and non-symptomatic (NS), Not significant (NS).

Cultivars R10 and R2 were found with mild to moderate symptoms, although both cultivars were resistant to almost all plants which varied from moderate to mild resistance respectively. R2 was homozygous resistant to*Ty-2* and *Ty-3* while R10 was resistant to*Ty-3*

and *Ty-5* (Table-1). These cultivars were characterized with mild leaf rolling, the newly emerging leaves with curling and vein thickening and leaf thickening. All infected plants of R7, R8 and R14 exhibited severe symptoms, which were characterized by leaf rolling, yellowing, vein thickening, growth stunting and enation an important feature of begomoviruses infection. Enation was not developed in all cultivars except for R7 and R8 cultivars. R15 which was homozygous susceptible for all genes and susceptible cultivars (Nagina) lacked a resistant gene which showed growth stunting, leaf yellowing, reduced leaf area, rolling and curling of leaves particularly newly emerging leaves, crumpled leaves and vein thickening (Fig-4).

R7 was homozygous and resistant to *Ty-2* and *Ty-3*. The R8 and R14 were resistant to *Ty-2*. There were however, variations in symptoms severity, as *Ty-3* had a major affect which accounted for 60% of the variance of symptoms severity (Ji *et. al.,* 2009), while *Ty-5* accounts for greater than 40%, it indicated *Ty-5* had less effect on resistance (Anbinder *et al.*, 2009). *Ty-3* tomato lines were found highly resistant against bipartite *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl palampur virus* (ToLCPalV) and monopartite *Tomato leaf curl Bangalore virus* (ToLCBV) reported by (Prasanna *et. al.*, 2015b). While *Ty-2* lines although showed moderate resistance against monopartite but were not effective against bipartite viruses (Prasanna *et. al.*, 2015b). However, a study conducted on *Ty*-3 line showed high resistance level against ToLCTWV, TYLCTHV and multiple bipartite begomoviruses (Garcia *et. al.*, 2008). It was further reported that *Ty*-3 line reduced the symptoms severity of TYLCD but was not very effective against this virus. *Ty*-3 and *Ty*-2 combination however could not eliminate virus infection (Hanson *et. al*., 2016). In study the present all three lines conferred complete resistance against virus infection to which these cultivars were exposed. Although these were unknown.

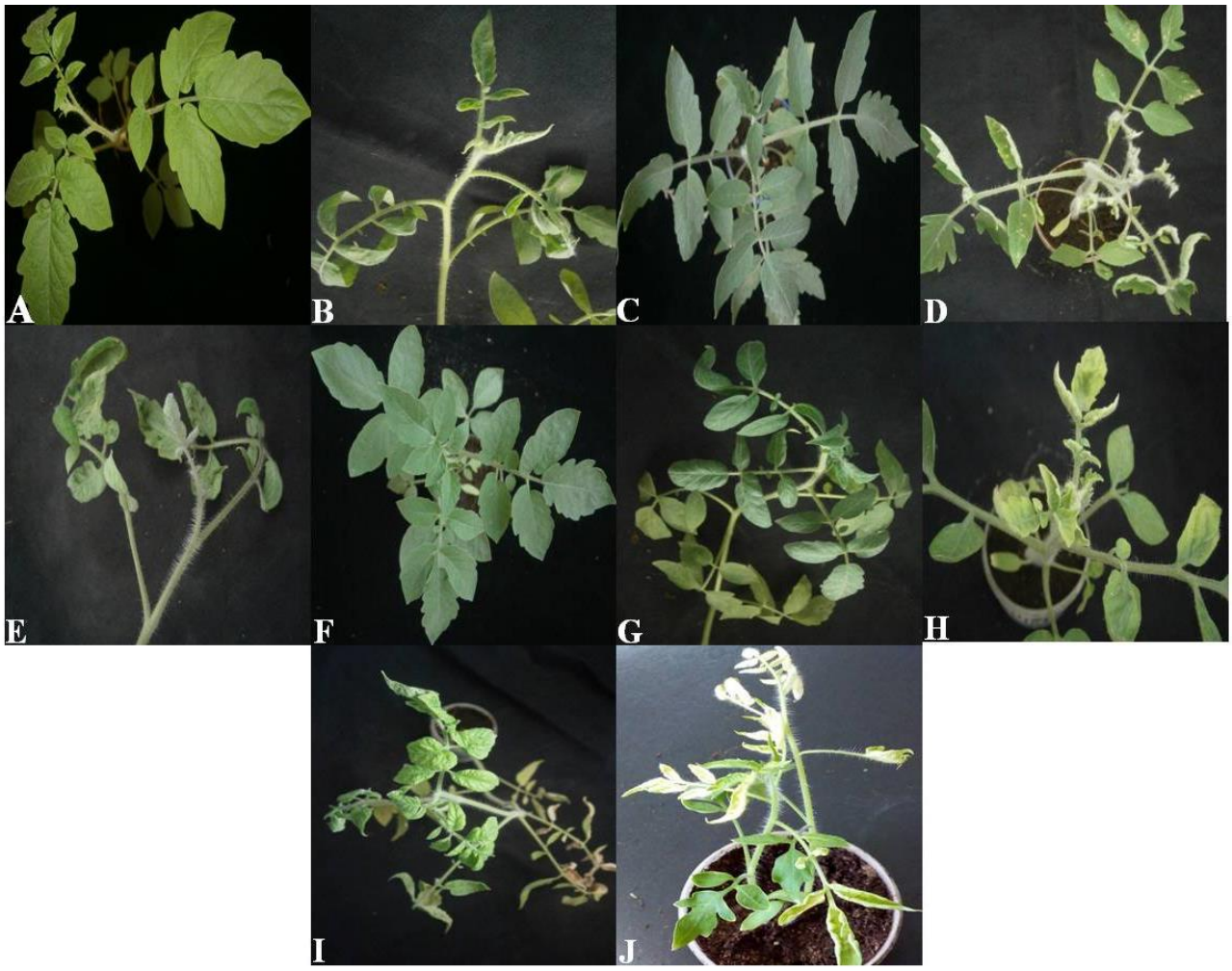

Fig-4: Symptoms variations in resistant and susceptible cultivars, R1 represented by figure (A), R6 (C) and R9 (F) were phenotypically normal. R2 (B) and R10 (G) showed moderate to mild symptoms, R7 (D), R8 (E), R14 (H) exhibited severe symptoms while R15 (I) and Nagina (J) showed very severe symptoms.

GCMS analysis of resistant sample (R6) indicated the presence of 44 compounds (Table-2). Among 44 identified compounds major were Pentadecane (2.70%), Tetradecane (6.77%), Heptadecane (5.64%), Tetradecane, 2, 6, 10-trimethyl (2.03%), Phenol, 3,5-bis(1,1-dimethylethyl)- (3.39%), Hexadecane, 2,6,11,15-tetramethyl- (2.03%), Hexadecane (6.77%), Pentadecane, 8-hexyl- (5.42%), Pentatriacontane (2.26%), Eicosane, 10-methyl- (3.9%), Tetrapentacontane (4.96%), Tetratetracontane (3.39%), Eicosane (7.22%), Hexatriacontane (8.58%), Tritetracontane (4.74%) and Docosanoic acid, docosyl ester (11.05%).Most of these compounds showed antimicrobial activities and contributed in plant defense mechanism (Kokate *et. al*., 2008) and were associated with resistance in plants against microbes or pests. Plant roots exudates influenced root-knot nematodes egg hatch, as root exudates of resistance tomato cultivars were known to produce higher amount of compounds associated with resistance, which included heptadecane, tridecane, tetradecane, hexadecane, pentadecane, hexatriacontane, hexadecane, 2,6,10,14-tetramethyl, octadecane, nonadecane, eicosane and heneicosane against *Meloidogyne incognita*(Yang *et. al.*, 2016). Similarly 2,6,10-trimethyldodecane (farnesane) terpenoid pathway-related parent compounds, for about 10,000 sesquiterpenes, induced in infected leaves (Choi *et. al*., 2008). In another study against *Phytoplasma* pathogenicity indicated metabolite changes caused in leaves and phloem sap leading to change in quantity of various compounds along with the following mentioned eicosane, tetradecane, 5-methyltetradecane, heneicosane, heptadecane, dodecane, hexatriacontane, pentatriacontane, tetrapentacontane, Tetratetracontane and 3,7-dimethyldecane (Gai *et. al.*, 2014).

Table-2.Gas Chromatography Mass Spectrometryanalysis of R6 cultivar.

Sr. No.	Retention Time	Compound name	Molecular Formula	Molecular Weight	Relative abundance percentage
1	6.28	p-Xylene	C8H10	106	0.07%
2	10.27	2,4,6-Trimethyloctane	C11H24	156	0.22%
3	11.05	Nonane, 2,6-dimethyl-	C11H24	156	0.07%
4	12.20	Undecane, 3,7-Dimethyl	C13H28	184	0.45%
5	13.86	Undecane	C11H24	156	0.45%
6	15.74	Silane, cyclohexyldimethoxymethyl-	C9H20O2Si	188	0.16%
7	16.29	Decane, 1-iodo-	C10H21I	268	0.16%
8	17.25	Dodecane	C12H26	170	1.80%
9	17.61	Undecane, 2,6-Dimethyl	C13H28	184	0.45%
10	18.33	Tridecane	C13H28	184	0.41%
11	18.63	Dodecane, 2,6,11-trimethyl	C15H32	212	0.45%
12	18.8	Heptadecane, 2,6,10,15-tetramethyl-	C21H44	296	0.67%
13	18.85	Undecane, 2,4-dimethyl	C13H28	184	0.31%
14	19.14	Pentadecane	C15H32	212	2.70%
15	19.54	Decane, 2,3,5,8-tetramethyl	C14H30	198	0.23%
16	19.61	Nonadecane	C19H40	268	1.13%
17	20.14	Dodecane, 2,6,10-trimethyl-	C15H32	212	0.90%
18	20.5	Tridecanol, 2-ethyl-2-methyl-	C16H34O	242	0.23%
19	20.76	Decane, 1,1 oxybis	C20H42O	298	0.25%
20	20.86	10-Methylnonadecane	C20H42	282	0.25%
21	20.97	7-Butyldocosane	C26H54	366	0.45%
22	21.1	2-Bromo dodecane	C12H25Br	248	0.41%
23	21.62	Tetradecane	C14H30	198	6.77%
24	21.85	Octadecane	C18H38	254	1.39%
25	21.95	Heneicosane, 11-(1-ethylpropyl)-	C26H54	366	0.68%
26	22.99	Heptadecane	C17H36	240	5.64%
27	22.93	Tetradecane, 2, 6, 10-trimethyl	C17H36	240	2.03%
28	23.31	Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O	206	3.39%
29	23.49	1-Chloroheptacosane	C27H55Cl	414	1.13%
30	23.65	Hexadecane, 2,6,11,15-tetramethyl-	C20H42	282	2.03%
31	24.5	1-Decanol, 2hexyl-	C16H34O	242	0.90%
32	24.59	Hexadecane	C16H34	226	6.77%
33	24.90	Octacosane	C28H58	394	1.92%
34	25.19	Hentriacontane	C31H64	436	1.81%
35	25.79	Tetratriacontane	C34H70	478	1.81%
36	25.83	Pentadecane, 8-hexyl-	C21H44	296	5.42%

37	26.36	Pentatriacontane	C35H72	492	2.26%
38	27.05	Eicosane, 10-methyl-	C21H44	296	3.9%
39	28.25	Tetrapentacontane	C54H110	758	4.96%
40	29.21	Tetratetracontane	C44H90	618	3.39%
41	30.4	Eicosane	C20H42	282	7.22%
42	32.55	Hexatriacontane	C36H74	506	8.58%
43	31.18	Tritetracontane	C43H88	604	4.74%
44	35.55	Docosanoic acid, docosyl ester	C44H88O2	648	11.06%

GCMS analysis of susceptible (Nagina) cultivar showed identification of 33 compounds (Table-3). Major compounds identified in Nagina sample were Dodecane (2.22%), 2-Bromo dodecane (3.45%), Hexadecane (5.43%), Octacosane (2.48%), Heneicosane (6.18%), Heptadecane (4.45%), Tetrapentacontane (11.74%), Pentatriacontane (7.66%), Tetratetracontane (8.89%), Tetracontane (7.97%), Hexatriacontane (11.37%), Tetrapentacontane, 1,54-dibromo (8.407%) and 1,2-Benzenedicarboxylic acid and ditridecyl ester (11.62%) given in(Table-3). GCMS analysis of susceptible cultivars indicated identification of less metabolites number than resistant cultivars, but major compounds contributed towards resistance as previous studies (Yang *et. al.*, 2016 and Gai *et. al.*, 2014) which showed more percentage values that might be due to plants response towards infections.

Table-3: Gas Chromatography Mass Spectrometry analysis of susceptible cultivar

Sr. No.	Retention Time	Compound name	Molecular Formula	Molecular Weight	Relative abundance percentage
1	5.53	Benzeneethanol, .alpha.,.beta.-dimethyl-	C10H14O	150	0.12%
2	6.69	p-Xylene	C8H10	106	0.06%
3	10.26	Undecane, 4,6-Dimethyl	C13H28	184	0.25%
4	10.71	Decane, 2,5,6-trimethyl	C13H28	184	0.06%
5	11.05	Heptane, 5-ethyl-2-methyl	C10H22	142	0.19%
6	11.5	1-Hexanol, 2-ethyl-	C8H18O	130	0.22%
7	12.21	5-Methyl undecane	C12H26	170	0.49%
8	12.41	Nonane, 4,5-dimethyl-	C11H24	156	0.25%
9	12.59	Nonane, 3-methyl	C10H22	142	0.18%
10	13.85	Dodecane	C12H26	170	0.44%
11	15.75	Silane, cyclohexyldimethoxymethyl-	C9H20O2Si	188	0.25%
12	16.29	3-Methylundecane	C12H26	170	0.198%
13	17.25	Dodecane	C12H26	170	2.22%
14	17.61	Undecane, 2,6-Dimethyl	C13H28	184	0.49%
15	17.85	4-Methyldodecane	C13H28	184	0.37%
16	18.33	Tetradecane	C14H30	198	0.49%
17	18.64	Dodecane, 4,6-dimethyl	C14H30	198	0.57%
18	18.87	Nonadecane	C19H40	268	1.24%
19	19	2-Bromo dodecane	C12H25Br	248	3.45%
20	19.73	Tridecane	C13H28	184	0.87%
21	20.14	Heptadecane, 2,6,10,15-tetramethyl-	C21H44	296	1.12%
22	21.62	Hexadecane	C16H34	226	5.43%
23	23.65	Octacosane	C28H58	394	2.48%
24	22.99	Heneicosane	C21H44	296	6.18%
25	23.31	Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O	206	0.35%
26	24.6	Heptadecane	C17H36	240	4.45%
27	30.40	Tetrapentacontane	C54H110	758	11.74%
28	29.45	Pentatriacontane	C35H72	492	7.66%
29	30.05	Tetratetracontane	C44H90	618	8.89%
30	28.25	Tetracontane	C40H82	562	7.97%
31	31.72	Hexatriacontane	C36H74	506	11.37%
32	35.1	Tetrapentacontane, 1,54-dibromo-	C54H108Br2	914	8.407%
33	35.5	1,2-Benzenedicarboxylic acid, ditridecyl ester	C34H58O4	530	11.62%

Most of the resistance sources however were known to sustain virus replication, but the virus accumulation level was quite less than the susceptible cultivar. It is well known about the *Ty-1/Ty-3* lines which contained 10% less virus titer than found in susceptible cultivars (Verlaan *et. al.*, 2013). The resistant cultivars also indicated positive correlation in amount of virus present and symptoms severity shown as has been reported by (Barbieri *et. al.*, 2010).

Pyramiding from two or three resistance genes combinations indicated either broader resistance spectrum or an increased level of resistance. Resistant genes pyramiding were affective in other plants-viruses interactions. Pyramiding for different plant-virus interaction included, study conducted by genes pyramiding in *Phaseolus vulgaris* against bean common mosaic virus was found more affective (Kelly *et. al*., 1995), similar results were found in other plant-virus interactions as in *Glycin max* against *Soybean mosaic virus* (SMV) reported by (Shi *et. al.*, 2009) and in *Hordeum vulgare* against *Barley mild mosaic virus* (BaMMV) as well as *Barley yellow mosaic virus* (BaYMV) (Werner *et. al.*, 2005)

However pyramiding for various plant-pathogens was also successful as in rice bacterial leaf blight disease caused by *Xanthomonas oryzae* (Suh *et. al.*, 2013). Bt genes pyramiding against insects *i.e., Spodoptera litura* and *Heliothis armigera* are also reported by (Li *et. al.*, 2014). Similarly in tomato, pyramiding strategy has been used for multiple disease resistance studies (Hanson *et. al.*, 2016).

Pyramidal lines response to natural infection indicated that ToLCD severity was higher in *Ty-1* and *Ty*-2 carrying lines, *Ty*-1 lines exhibited more disease severity and incidence in fields, as mixed infection were common to natural conditions (Verlaan *et. al.*, 2013).

However field screening for tomato yellow leaf curl disease under high pressure, *Ty* resistant lines exhibited high resistance (Hanson *et. al*., 2016). Begomoviruses being highly divergent due to migration and recombination, consequently leading to gene pyramiding strategy by combining multiple resistant genes in order to confer durable resistance against these diseases (Ji *et. al.*, 2008, Nowicki *et. al.*, 2012 and Vidavski *et. al.*, 2008).

Among all pyramidal combinations, *Ty*-2, *Ty*-3 and *Ty*-5 genes harboring cultivars were more resistant and conferred better resistance than *Ty*-1/*Ty*-3 genes carrying cultivars. However, due to mixed infections which caused synergism and led to symptoms severity, some variation in response could occur. GCMS analysis of resistant and susceptible cultivars indicated presence of numerous bioactive compounds or metabolites associated with resistance.

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