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ARTICLE

Evaluation of Tomato Hybrids for Resistance against Tomato Mosaic Virus (ToMV)

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ABSTRACT

Tomato mosaic virus (ToMV) drastically affects the tomato production worldwide. To deal with this problem, breeding of ToMV-resistant hybrids/varieties is the ultimate need and most successful approach. In wild tomato species, three dominant ToMV-resistant genes (*Tm-1*, *Tm-2* and *Tm-2*^{2}) were identified and the World Vegetable Center developed few fresh market tomato lines resistant to ToMV by the introgression of these genes. Recently at Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan a breeding programme was initiated to develop high yielding and ToMV tolerant hybrids using these lines. Current study was performed to screen elite F_1 hybrids carrying *Tm* gene along with their parents against ToMV using mechanical inoculation, confirmation of the virus using DAS-ELISA and marker assisted selection of hybrids. Out of 28 hybrids and 17 parent accessions/genotypes, eight hybrids and five accessions were found to be highly resistant and the virus was not detected in DAS-ELISA. Five hybrids were resistant, nine hybrids and four genotypes were tolerant, while the remaining six hybrids and eight genotypes were susceptible. For the confirmation of *Tm-2*^{2} gene, the tomato hybrids and their parents were subjected to molecular analysis using cleaved amplified polymorphic sequence (CAPS) primers. The result of CAPS markers for the confirmation of *Tm-2*^{2} gene was found consistent with phenotypic data of the inoculated tomato genotypes/hybrids. Higher phenolic content, total soluble proteins, better CAT and SOD activities were positively correlated with resistance. Screening results based on phenotype, biochemical and molecular marker data indicate that hybrids carrying *Tm-2*^{2} gene are good sources of resistance against ToMV.

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1. Introduction

Tomato (*Solanum lycopersicum L.*) is the second most produced and consumed vegetable in the world, but face several biotic stresses. Among 136 viruses infecting tomato [1], *Tomato Mosaic Virus* (ToMV) is one of the highly stable, contagious, cosmopolitan tobamovirus and is particularly problematic for greenhouse tomato production [2,3]. ToMV infected tomato plants show wrinkles, light green or yellow mottling, curved leaves, shoestring, stunted growth with irregular ripening of fruits [4,5]. To overcome ToMV problem in tomato, resistant variety is the most desirable and practical approach [2]. In wild tomato species, three dominant ToMV-resistant genes (*Tm-1*, *Tm-2* and *Tm-2²*) were identified [6] and have been used to incorporate resistance in cultivated tomato [7]. These resistant genes inhibit viral replication, hence increasing durability of crops [8]. The *Tm-1* gene was originally identified in *S. habrochaites* and is incompletely dominant gene, while both *Tm-2* and *Tm-2²* are dominant genes identified in *S. peruvianum* [7]. *Tm-1* gene is present on chromosome 2 [9], while genes *Tm-2* and *Tm-2²* are located on chromosome 9. Among these genes, *Tm-2²* is the most effective and durable *R* gene [10] and provides resistance against all the three known strains of ToMV (0, 1 and 2) [11]. *Tm-2²* confers resistance by recognizing ToMV movement proteins [12]. For *Tm* genes confirmation in tomato, several markers were developed and used [13,14]. DNA-based molecular markers linked to resistant genes are promising tools without recording phenotypic data and hence reduces the time and cost involved in conventional approaches [5]. Recently, for *Tm-2²* gene confirmation, an efficient and robust CAPS marker were reported by Panthee, D. R. et al. [5],which can successfully identify tomato genotypes carrying *Tm-2²* resistant gene [2]. The World Vegetable Center had developed few fresh market tomato lines resistant to ToMV by the introgression of *Tm-2²*. Our previous study confirmed the resistance level of these *Tm-2²* harboring accessions against Pakistani isolate of ToMV [2]. Based on our finding, we initiated a breeding programme at Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan (NIAB) to develop high yielding and ToMV tolerant tomato hybrids. In this study, we analyzed the elite F_1 hybrids developed through hybridization with *Tm-2²* parent accessions against ToMV using mechanical inoculation in insect-free glass-house. We further identified *Tm-2²* presence in resistant hybrids using molecular markers linked to this gene. In this study we also determine the biochemical alterations in selected six resistant and susceptible hybrids following ToMV inoculation.

2. Materials and Methods

2.1 Inoculation and Screening

The ToMV isolate (MG975645) used in this study was described in Ullah et al. [2] and maintained on susceptible tomato variety "Riogrande" in an insect-proof glasshouse. Plant material comprising five *S. lycopersicum* accessions (NB-324, NB-327, NB-328, NB-333, NB-336) harboring *Tm-2²* gene, 12 cultivated genotypes without *Tm-2²* gene and 28 hybrids developed at Nuclear Institute for Agriculture and Biology (NIAB) Faisalabad, Pakistan through hybridization of *Tm-2²* gene carrying accessions and cultivated genotypes. Nursery seedling of each genotype was raised in pots under an insect-proof glasshouse. One month post germination, plants were thinned into four plants per pot. At the age of six week, test plants were mechanically inoculated keeping variety "Nagina" as susceptible control. Top fully expanded leaves pre-dusted lightly with carborundom were inoculated with ToMV infected leaves sap (triturated in phosphate buffer; 0.01 M, pH 7) and subsequently washed after 10 min with distilled water. Plants were kept in good condition by adopting normal agronomic practices. Experimental unit was observed daily and data were recorded as the percentage of disease transmission, latent period and disease severity using the modified rating system given in Table 1 [15]. Leaf sample from each tomato genotype was collected and tested for ToMV presence using Double Antibody Sandwiched Enzyme-Linked ImmunoSorbant Assay (DAS-ELISA) [3]. For this purpose polyclonal antibodies (V061-K1, ACD, Inc.) were used according to the protocol of the manufacturer (ACD, Inc.). Results were assessed by visual observation and spectrophotometric absorbance at 405 nm. Samples were considered as positive if the optical density (OD) at 405 nm was at least twice of the negative control [9]. Total DNA from tomato hybrids and genotypes, plant samples were extracted using CTAB (Cetyltrimethylammonium Bromide) method. The extracted DNA was used as template in PCR using cleaved amplified polymorphic sequence primers (CAPS). Each 25 µL of PCR reaction mixture consist of 12.5 µL PCR Master Mix (enzynomics, Korea), 0.5 µL each *NCTm-019* 5′-AATTTGGGCATACTGACATC-3′ and 5′-GTTGCACACATTGGTTGTAG-3′ primers, 2 µL DNA template and 9.5 µL nuclease free water. Thermal profile was set for initial denaturation at 94 °C for 5 min, followed by 35 cycles (denaturation at 94 °C for 45 s, annealing at 54 °C for 1 m, extension at 72 °C for 1.5 min) and final extension at 72 °C for 10 min. PCR products were first separated on 1.5% agarose gel, stained with EtBr and DNA bands were observed using gel documentation system (UVIpro). After amplification

with CAPS primers, the obtained PCR products were restricted using HaeIII enzyme (Cat. No R0108S, New England Biolabs) at 37 °C for 1 hour. For enzyme digestion, 25 µL reaction mixture (8 µL PCR product, 2.5 µL of 10x enzyme buffer, 1 µL HaeIII enzyme and 13.5 µL distilled water) was prepared. Digested PCR products were separated on 2% agarose gel, stained with EtBr and observed using gel documentation system (UVIpro).

Table 1. Disease scale rating for mosaic disease in tomato caused by ToMV

Rating	Symptoms	Severity index	Disease reaction
0	No visible disease symptoms. Virus can't be detected in plant tissues via molecular techniques.	0	Highly resistant
1	Complete absence of symptoms but virus can be detected in plant tissues using moleculartechniques. OR Slight mosaic appearance or mottling and leaf deformity but no shoe stringing.	0.01-1.4	Resistant
2	Moderate mosaic appearance or mottling and leaf deformity followed by minor shoe stringing.	1.5-2.4	Tolerant
3	Severe mosaic or mottling. Leaf deformity, shoe-stringing, minor to medium stunting with minor flower shedding and minor reduction in fruit setting but marketable fruit setting.	2.5-3.4	Susceptible
4	Severe mosaic or mottling, leaf deformity, shoe-stringing, stunting with no or few unmarketable fruit setting.	3.5-4.0	Highly susceptible

2.2 Biochemical Parameters

To study the biochemical changes occurred as a result of ToMV infections in six resistant and susceptible tomato hybrids, leaf samples were ground in extraction buffer. After grinding, the mixture was brought to centrifugation at maximum speed (14,000 rpm) for 10 minutes. Following centrifugation, supernatant was removed and samples were stored at –20 °C to study various antioxidant and enzymatic activities.

Total phenolic contents (TPC) were estimated among virus-inoculated and non-inoculated plants as described by Ainsworth and Gillespie (2007) [22]. A standard curve was prepared using different concentration of gallic acid and a linear regression equation was calculated to determine TPC. Total soluble protein (TSP) contents were measured using Bradford's method [23]. For catalase (CAT) estimation, procedure described by Beers and Sizer [24] was followed. Superoxide dismutase (SOD) activity was estimated as described by Dixit et al. [25]. The level of lipid peroxidation was measured in terms of Malondialdehyde (MDA), a product of lipid peroxidation) [26]. Estimation of pigments like chlorophyll (Chl. *a* and *b*), total chlorophyll, lycopene and carotenoids was done following the method of Hameed et al. [27].

2.3 Statistical Analysis

Significance level of data was calculated by ANOVA and Tukey's (HSD) test at $p \leq 0.05$ by using XL-STAT software. Mean ± S.E values are shown in graphs with different alphabets that differ significantly from each other.

3. Results

3.1 Evaluation of Hybrids and Parents

ToMV was successfully transmitted to 28 hybrids and 17 parent accessions/genotypes belonging to *S. lycopersicum* using mechanical inoculation. ToMV infection in these hybrids and parent accessions/genotypes was confirmed based on their phenotpes and following DAS-ELISA. Eight hybrids (NBH-148, NBH-149, NBH-151, NBH-174, NBH-175, NBH-200, NBH-256 and NBH-260) and 5 accessions (NB-324, NB-327, NB-328, NB-333 and NB-336) were found to be highly resistant as no disease symptoms were observed on these hybrids/accessions till the end of experiment i.e., 90 days post inoculation (Tables 2 and 3). However, 5 hybrids (NBH-154, NBH-204, NBH-258, NBH-259 and NBH-261) showed a much greater degree of resistance (with slight mosaic appearance or mottling and leaf deformity) with the latent period (LP) ranging between 13-18 days, severity index (SI) value of 1.0 to 1.3 and low virus titer through ELISA (0.82 to 0.97) with no reduction in disease severity throughout the experiment (Table 2).

Nine hybrids (NBH-150, NBH-152, NBH-227, NBH-229, NBH-263, NBH-265, NBH-266, NBH-267 and NBH-268) and four genotypes (NB-8, NB-10, NB-279 and NB-299) were tolerant with SI value of 1.7 to 2.3, LP ranging between 14-18 days and low virus titer through ELISA (1.60 to 2.14) as compared to susceptible hybrids/accessions. Remaining six hybrids and eight genotypes were susceptible. ToMV symptoms started as mild mosaic or mottling and leaf deformity from 11 to 12 days post inoculation in all susceptible hybrids and genotypes. All these genotypes developed severe mosaic or mottling, and leaf deformity, shoe-stringing, minor to medium stunting at 30 days to post inoculation with SI ranging from 2.7 to 3.3 with virus titer ranging from 2.37 to 3.50 (A_{405nm} 1 h) (Tables 2 and 3).

Table 2. Tomato hybrids evaluated against ToMV in an insect-proofed glasshouse.

Sr. No	Hybrid	Pedigree	Habit	Latent period	Disease severity	Disease response	ELISA		PCR confirmation of Tm-2^2 (–/+)
							–/+	Values*	
1	NBH-147	NB-11×NB-327	D	11	3.0	Susceptible	+++	3.50	–
2	NBH-148	NB-11×NB-333	D	-	0.0	Highly resistant	–	0.57	+
3	NBH-149	NB-242×NB-327	D	-	0.0	Highly resistant	–	0.59	+
4	NBH-150	NB-242×NB-333	D	14	2.3	Tolerant	++	1.85	+
5	NBH-151	NB-8×NB-327	D	-	0.0	Highly resistant	–	0.59	+
6	NBH-152	NB-242×NB-327	D	14	2.0	Tolerant	++	1.90	+
7	NBH-154	NB-242×NB-333	D	17	1.3	Resistant	+	0.90	+
8	NBH-174	NB-328×NB-285	D	-	0.0	Highly resistant	–	0.61	+
9	NBH-175	NB-285×NB-328	D	-	0.0	Highly resistant	–	0.58	+
10	NBH-196	NB-216×NB-327	D	11	3.3	Susceptible	+++	3.36	–
11	NBH-200	NB-243×NB-327	D	-	0.0	Highly resistant	–	0.58	+
12	NBH-204	NB-279×NB-327	D	15	1.3	Resistant	+	0.82	+
13	NBH-227	NB-11×NB-336	D	14	2.3	Tolerant	++	1.87	+
14	NBH-228	NB-242×NB-336	D	12	3.0	Susceptible	++	2.18	–
15	NBH-229	NB-8×NB-336	D	17	2.3	Tolerant	++	1.96	+
16	NBH-255	PRN×NB-324	ID	12	3.0	Susceptible	+++	3.25	–
17	NBH-256	PRN×NB-333	ID	-	0.0	Highly resistant	–	0.60	+
18	NBH-257	PRN×NB-336	ID	12	3.0	Susceptible	+++	2.90	–
19	NBH-258	NB-10×NB-324	D	18	1.3	Resistant	+	0.93	+
20	NBH-259	NB-10×NB-333	D	17	1.0	Resistant	+	0.97	+
21	NBH-260	NB-10×NB-336	D	-	0.0	Highly resistant	–	0.57	+
22	NBH-261	NB-210×NB-324	D	13	1.3	Resistant	+	0.87	+
23	NBH-263	NB-210×NB-336	D	17	1.7	Tolerant	+++	2.14	+
24	NBH-265	NB-242×NB-32	D	16	2.3	Tolerant	++	2.09	+
25	NBH-266	NB-279×NB-324	D	16	2.3	Tolerant	++	2.20	+
26	NBH-267	NB-279×NB-333	D	15	2.3	Tolerant	+++	1.86	+
27	NBH-268	NB-14×NB-324	D	13	2.3	Tolerant	+++	1.94	+
28	NBH-281	NB-8×NB-324	D	12	2.7	Susceptible	+++	3.30	–

D= determinate; ID= indeterminate: * = ELISA absorbance values (A405 nm) after 1h: ELISA -/+ = - is absence of ToMV/ Tm-2^2 gene and + is presence of ToMV/ Tm-2^2 gene

Table 3. Tomato accessions/genotypes evaluation against ToMV using sap-inoculation under glasshouse.

Sr. No	NIAB code	Habit/ Source	Latent period	Severity index	Disease response	ELISA		PCR confirmation of Tm-2^2 (–/+)
						–/+	Values*	
1	NB-324	D/AVRDC	-	0	Highly resistant	+	0.83	+
2	NB-327	SD/AVRDC	-	0	Highly resistant	+	0.77	+
3	NB-328	SD/AVRDC	-	0	Highly resistant	+	0.87	+
4	NB-333	D/AVRDC	-	0	Highly resistant	+	0.86	+
5	NB-336	D/AVRDC	-	0	Highly resistant	+	0.76	+
6	NB-8	D/AARI	15	2.3	Tolerant	++	1.60	–
7	NB-10	D/Bulgaria	16	2.0	Tolerant	++	1.90	–
8	NB-11	ID/Bulgaria	15	3.0	Susceptible	+++	3.50	–
9	NB-210	D/Bulgaria	12	3.3	Susceptible	+++	2.89	–
10	NB-216	D/EFUP	13	2.3	Susceptible	+++	2.93	–
11	NB-242	D/TGRC	12	3.3	Susceptible	+++	3.49	–
12	NB-243	D/USA	12	3.3	Susceptible	+++	3.26	–
13	NB-260	D/AARI	13	3.3	Susceptible	+++	3.10	–
14	NB-279	D/GWP	18	2.3	Tolerant	++	1.84	–
15	New Yorker	D/TGRC	12	3.3	Susceptible	+++	2.37	–
16	NB-299	D/TGRC	16	2.3	Tolerant	++	1.88	–
17	PRN	ID/?	13	3.3	Susceptible	+++	2.90	–

* = ELISA absorbance values (A405nm) after 1h: ELISA -/+ = - is absence of ToMV/ Tm-2^2 gene and + is presence of ToMV/ Tm-2^2 gene; D= determinate; ID= indeterminate; SD= semi-determinate; AARI= Ayub Agricultural Research Institute, Faisalabad, Pakistan; TGRC= Tomato Genetic Resources Centre, United States of America; AVRDC= Asian Vegetable Research and Development Centre, Taiwan; EFUP= Establishment of facilitation unit for participatory vegetable seed and nursery production programme, Pakistan; GWP= Gujranwala Pakistan.

3.2 Molecular Analysis for the Identification of *Tm-2^2* Gene

After defining the resistance/susceptibility criteria in the hybrids and their respective parent genotypes through mechanical inoculation, tomato hybrids and their parent were subjected to molecular methods for further confirmation. The CAPS marker was used for the confirmation of *Tm-2^2* gene. CAPS markers efficiently differentiated the resistant hybrids and their parent from the susceptible ones. PCR products of 870 bp was successfully amplified for all the tomato hybrids and their parent tested against ToMV. However, when the CAPS primers amplified products of susceptible hybrids and their parent were digested with HaeIII restriction enzyme, it produced a single uncleaved intact band of 870 bp which showed the absence of *Tm*-2^2 gene in these hybrids and their parent. Conversely, when the highly resistant/resistant hybrids PCR products were restricted, it showed three different bands of 870 bp, 600 bp and 270 bp. These results shows the presence of *Tm-2^2* gene in these hybrids in heterozygous conditions. HaeIII restricted PCR product of highly resistant parent genotypes showed two bands of 600 bp and 270 bp, which confirmed the presence of *Tm-2^2* gene in these genotypes in homozygous conditions (Tables 2 and 3). Furthermore, result of CAPS markers digested with HaeIII was found consistent with phenotypic data of the inoculated tomato genotypes/ hybrids.

4. Biochemical Analysis

In the present study, the level of TPC in ToMV-inoculated plants of all the tested hybrids was differed significantly within highly resistant, resistant and susceptible classes (Table 4). TPC were higher in highly resistant hybrid NBH-149, while lower in susceptible hybrid NBH-268. TPC values for tomato hybrids viz., NBH-149, NBH-151, NBH-265, NBH-154, NBH-196 and NBH-268 after inoculation with ToMV were 925.0 µM/g.f.wt., 825.0 µM/g.f.wt., 397.5 µM/g.f.wt., 352.5 µM/g.f.wt., 246.0 µM/g.f.wt., and 165.0 µM/g.f.wt., respectively.

Amount of TSP in all the highly resistant, resistant and susceptible classes was differed significantly (Table 4). TSP content was more in resistant hybrids as compared to susceptible ones. Its amount was particularly high in hybrids NBH-154, NBH-149 and BH-265 than other hybrids. Moreover, significant differences were observed between highly resistant (NBH-149 and NBH-151) and susceptible hybrids (NBH-196 and NBH-268). TSP values for tomato hybrids viz., NBH-154, NBH-149, NBH-265, NBH-151, NBH-196 and NBH-268 after inoculation with ToMV were 153.3 mg/g.f.wt., 143.7 mg/g.f.wt., 135.5 mg/g.f.wt., 101.5 mg/g.f.wt., 79.3 mg/g.f.wt. and 47.0 mg/g.f.wt., respectively.

Hybrids tested for catalase activity showed significant differences among all the three classes: highly resistant, resistant and susceptible (Table 4). Level of catalase was higher in resistant hybrid NBH-265, whereas least in susceptible hybrid NBH-196. Catalase level was significantly different among the classes. In the resistant class, NBH-265 and NBH-154 were statistically different from each other. Also, in the susceptible ones, NBH-196 and NBH-268 were statistically different. Catalase values for tomato hybrids viz., NBH-265, BH-151, NBH-149, NBH-154, NBH-268 and NBH-196 after inoculation with ToMV were 572.5 units/g.f.wt., 430.0 units/g.f.wt., 385.0 units/g.f.wt., 285.5 units/g.f.wt., 235.0 units/g.f.wt. and 62.5 units/g.f.wt., respectively.

Hybrids subjected to superoxide dismutase activity showed that they were significantly different among classes but insignificant between classes (Table 4). Highest SOD activity was observed in NBH-151 (highly resistant) and least in NBH-268 (susceptible). However, results were insignificant between highly resistant, resistant and susceptible categories. SOD values for tomato hybrids viz., NBH-151, NBH-149, NBH-196, NBH-265, NBH-154 and NBH-268 after inoculation with ToMV were 293.75 units/g.f.wt., 247.64 units/g.f.wt., 224.19 units/g.f.wt., 206.82 units/g.f.wt., 193.05 units/g.f.wt. and 106.15 units/g.f.wt., respectively.

In the present study, level of malondialdehyde in ToMV-inoculated plants of all the tested hybrids was insignificant within highly resistant, resistant and susceptible classes (Table 4). Statistical trend was similar in all the members of hybrid categories except NBH-196 that showed slight significance in its behaviour. MDA content in NBH-196 recorded 330.38 µM/g f.wt. MDA level in other hybrids viz., NBH-268, NBH-151, NBH-149, NBH-154 and NBH-265 was 239.16 µM/g.f.wt., 218.48 µM/g.f.wt., 216.2 µM/g.f.wt., 182.0 µM/g.f.wt. and 176.29 µM/g.f.wt., respectively.

Chlorophyll *a* (Chl a) level in ToMV-inoculated plants of all the tested hybrids recorded insignificant differences among highly resistant, resistant and susceptible categories (Table 4). Chl *a* was higher in resistant hybrid NBH-154, whereas least in susceptible hybrid NBH-268. Chl *a* values for tomato hybrids *i.e.,* NBH-154, NBH-149, NBH-151, NBH-196, NBH-265 and NBH-268 after inoculation with ToMV were 520.28 µg/g.f.wt., 507.66 µg/g.f.wt., 507.55 µg/g.f.wt., 505.69 µg/g.f.wt., 501.12 µg/g.f.wt. and 497.52 µg/g.f.wt., respectively.

Level of Chl *b* recorded in tomato genotypes proved insignificant among the tested hybrids (Table 4). Chl *b* was higher in highly resistant hybrid NBH-149, whereas least in resistant hybrid NBH-154. However, the trend of resistant hybrids was generally different from highly resistant and susceptible hybrids. Chl *b* values for tomato hybrids *i.e.*, NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 614.64 µg/g.f.wt., 597.23 µg/g.f.wt., 592.5 µg/g.f.wt., 563.61 µg/g.f.wt., 528.74 µg/g.f.wt. and 492.96 µg/g.f.wt., respectively. After calculating chlorophyll *a* and *b* level in ToMV-inoculated hybrids, total chlorophyll was also determined in all classes (highly resistant, resistant and susceptible). Results were statistically insignificant with NBH-149 being highest in total chlorophyll content and NBH-154 being lowest. Chlorophyll (a+b) values for tomato hybrids in NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 1122.31 µg/g.f.wt., 1104.79 µg/g.f.wt., 1098.19 µg/g.f.wt., 1061.13 µg/g.f.wt., 1029.86 µg/g.f.wt. and 1013.23 µg/g.f.wt., respectively (Table 4).

Level of lycopene in ToMV-inoculated plants of all the tested hybrids recorded nonsignificant differences among highly resistant, resistant and susceptible classes (Table 4). Lycopene was higher in highly resistant hybrid NBH-149, whereas lower in resistant hybrid NBH-265. Lycopene values for tomato hybrids *i.e.*, NBH-149, NBH-151, NBH-268, NBH-196, NBH-154 and NBH-265 after inoculation with ToMV were 18.31 mg/g.f.wt., 17.72 mg/

Table 4. Summary of comparison of different biochemical parameters in tomato hybrids inoculated with ToMV.

Parameter (g.f.wt.)	Hybrids					
	NBH-149	NBH-151	NBH-265	NBH-154	NBH-196	NBH-268
TPC (µM)	925^{a}	825^{a}	397.5^{b}	352.5^{bc}	246^{cd}	165^{d}
TSP (mg)	143.7^{a}	101.5^{b}	135.5^{a}	153.3^{a}	79.3^{b}	47.0^{c}
Catalase (units)	385.0^{b}	430.0^{b}	572.5^{a}	285.5^{c}	62.5^{e}	235.0^{d}
SOD (units)	247.64^{ab}	293.75^{a}	206.82^{bc}	193.05^{c}	224.19^{bc}	106.15^{d}
MDA (µM)	216.2^{b}	218.5^{b}	176.3^{b}	182.0^{b}	330.4^{a}	239.2^{b}
Lycopene (mg)	18.31^{a}	17.72^{a}	15.82^{a}	16.11^{a}	16.36^{a}	16.40^{a}
Chl *a* (µg)	507.66^{a}	507.55^{a}	501.12^{a}	520.28^{a}	505.69^{a}	497.52^{a}
Chl *b* (µg)	614.64^{a}	597.23^{a}	528.74^{a}	492.96^{a}	592.50^{a}	563.61^{a}
Total chl. (µg)	1122.31^{a}	1104.79^{a}	1029.86^{a}	1013.23^{a}	1098.19^{a}	1061.13^{a}
Carotene (mg)	45.47^{a}	45.24^{a}	43.95^{a}	45.24^{a}	45.09^{a}	42.71^{a}

Mean sharing similar letters in the same box do not differ from each other at p ¼ 0.05.

g.f.wt., 16.4 mg/g.f.wt., 16.37 mg/g.f.wt., 16.11 mg/g.f.wt. and 15.82 mg/g.f.wt., respectively.

Carotene level in all the ToMV inoculated hybrids proved to be insignificant within highly resistant, resistant and susceptible groups. Highest carotene level in NBH-149 and lowest in NBH-268 were observed (Table 4). Carotene values for tomato hybrids in NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 45.47 mg/g.f.wt., 45.24 mg/g.f.wt., 45.24 mg/g.f.wt., 45.09 mg/g.f.wt., 43.95 mg/g.f.wt. and 42.71 mg/g.f.wt., respectively (Table 4).

5. Discussion

ToMV being a highly infectious, contagious and rapidly multiplying virus, significantly reduces tomato yield [23]. Several measures were reported to manage this disease but the development and use of resistant variety are the most practical approach [2]. We tested 28 tomato hybrids and 17 parent genotypes against ToMV following mechanical inoculation. Wide variations were observed in results, from highly resistant to susceptible ones. Of these, 20 hybrids and 12 parent genotypes displayed a range of phenotypic reaction to ToMV infection. Besides their phenotypic response, ToMV presence was confirmed using DAS-ELISA. Eight hybrids and five accessions were symptomless. However, on five hybrids slight mottling and leaf deformity symptoms were recorded and they were rated as resistant with low ELISA absorbance value (A405 nm value; 0.87 to 0.97) compared to highly susceptible genotypes. Nine hybrids and four genotypes were tolerant and showed moderate level of symptoms. Rest of the six hybrids and eight parent genotypes were susceptible. In resistant hybrids, delayed symptoms development and low virus titer was observed (based on ELISA absorbance value). In contrast, susceptible hybrids were severely infected and showed high virus titer in ELISA. Severe mosaic, mottling, stunting and leaf deformation were noticed on susceptible hybrids/genotypes after ToMV inoculation. ToMV can induce different symptoms depending on susceptibility of genotype infected [3]. Level of disease severity can serve as an indicator of resistance level of a plant species against a pathogen [19-23]. Resistant varieties contain low viral titer, while the susceptible genotypes accumulate high virus titer [25-27]. Low viral titer accumulates in highly resistant genotypes [26], while in susceptible genotypes severe symptoms developed and facilitated high viral titer [25-28].

In this study we further tested the tomato hybrids for the confirmation of *Tm-2*^{2} gene using CAPS primers. A PCR product of 870 bp was successfully amplified from all the tomato hybrids and genotypes tested against ToMV. However, when the CAPS primers (NCTm-019) amplified products of susceptible genotypes were digested with HaeIII enzyme, a single un-cleaved intact band of 870 bp was observed, which showed the absence of *Tm-2*^{2} gene. Conversely, when the highly resistant/resistant hybrids PCR products were restricted, they showed three different bands of 870 bp, 600 bp and 270 bp.

These results show the presence of *Tm-2²* gene in these hybrids in heterozygous conditions. Two bands of 600 bp and 270 bp were observed when PCR product of highly resistant parent accessions were restricted with HaeIII enzyme. Appearance of two bands after restriction confirms the presence of *Tm-2²* gene in these accessions in homozygous conditions. Furthermore, CAPS markers digested with HaeIII enzyme were found consistent with phenotypic data of the inoculated tomato genotypes/hybrids. All the inoculated plants of highly resistant tomato hybrids and lines harboring *Tm-2²* resistant gene were symptomless even at the end of the experiment. These genotypes were also negative for ToMV infection tested in DAS-ELISA. A number of markers have been reported for the confirmation of *Tm* genes in tomato [13,14]. However, a recently reported sequence-based CAPS marker (*NCTm-019)* is found more efficient, robust and specific to *Tm-2²* gene [5]. Moreover, this marker clearly differentiate ToMV resistant genotypes carrying *Tm-2²* from susceptible ones on molecular level [2]. The sequence-based markers are more efficient than markers from flanking region [5]. Our results further confirm that CAPS marker NCTm-019 is a reliable, robust marker and its results are consistent with phenotypic response of the tested genotypes.

In the present study we also investigated that how appearance of symptoms relates to biochemical alterations. Phenols are compounds with well-known antifungal, antibacterial and antiviral properties that occur in plants and play a vital role in defense by enhancing the mechanical strength of host cell walls by the synthesis of lignin and suberin, both of which are involved in the formation of physical barriers that block the spread of pathogens. High levels of phenols is correlated with increased resistance in plants [29]. Our results concerning the criterion proved that amount of phenols in highly resistant and moderately resistant hybrids were significantly higher as compared to the amount of phenols in the susceptible hybrids. A positive correlation exists among the host resistance, total phenols and increased enzyme activities. However, it was exact opposite among susceptible hybrids. The positive relation between phenols and increased resistance could be of great significance for identification of resistant hybrids during screening of large populations [30]. Another such example of positive correlation was found in grape leaves and black currants presented by Vagiri et al. [31].

Many plant-pathogen interactions have shown the involvement of proteins and its components in plant disease resistance. Stimulation of defense proteins make the plants resistant to pathogens. Usually, infected plants show high levels of proteins that may be because of activation of host defense system or pathogen attack mechanism [32]. In our study, variable trend was seen in the protein contents of healthy plants. Resistant genotypes have shown increased levels of proteins, whereas significant decrease was observed in susceptible hybrids. A possible clarification for this significant decrease after infection may be because of high level of susceptibility of these hybrids [20]. Low level of soluble proteins might be possibly due to decrease in synthesis of ribulose-1,5-bisphosphate carboxylase (RuBPC); a major soluble protein of leaf. Loss of leaf protein could be because of damaged chloroplast or inhibition of protein synthesis [33].

CAT is an oxygen-scavenger, which protects cells from the toxic effects of substrates (H_2O_2) during development, which could be fatal otherwise [33]. In our study, CAT activity significantly increased within moderately resistant cultivars, whereas a significant decrease was observed among the hybrids of susceptible class. Usually, the reduction of CAT increases resistance in plants against pathogenic attack as plants can maintain high concentrations of H_2O_2 [29]. Reduction in CAT activity could be a result of increased proteolysis. On the other hand, higher CAT levels may be linked with decrease in H_2O_2 level and in lipid peroxidation [35].

Superoxide dismutase is another scavenger enzyme that catalyzes the dismutation of superoxide radicals to active oxygen species. Enhanced SOD activities were observed in resistant hybrids, but variable trend was seen in susceptible class. SOD activity in one of the susceptible hybrid NBH-196 was significantly different (higher) from NBH-268. Summing up, resistant is associated with SOD activity. In another study performed on strawberry leaves that were infected with *Mycosphaerella fragariae*, confirmed that SOD was higher in resistant genotypes than the susceptible ones [34]. Research conducted by Lu et al., also confirmed that resistance is positively related to the increase in SOD activities [36].

Malondialdehyde (MDA) is an abundant aldehydic lipid breakdown product. MDA acts as a secondary messenger that up-regulates several genes in plants under stress conditions [37]. MDA produced during lipid peroxidation acts as an indicator to measure extent of cellular damage as a result of pathogenic infection. MDA levels determined were statistically insignificant (similar) among all the highly resistant, moderately resistant and susceptible classes except NBH-196 that showed an irregular increase in MDA levels. This represents that MDA content has nothing to do with resistance of plants. The same concept was also confirmed by Siddique et al., where MDA was statistically similar in all resistant and susceptible varieties [34].

The photosynthetic system (chlorophyll a + b) is the

physiological basis of plant growth and crop production. Any kind of environmental stress affecting photosynthetic system will directly affect plant growth. In our present study, chlorophyll (a and b) rates remain totally unaffected and their differences proved statistically insignificant. However, Chl *b* level recorded was higher than Chl *a* in the inoculated genotypes. This is due to the reason that Chl *b* is involved in the virus tolerance mechanism. Similar insignificant results were also presented by Siddique et al. [38].

Lycopene is a pigment that gives red or orange-red colour to fruits and vegetables. Lycopene acts a free-radical scavenger and have antioxidant properties [39]. Evaluation of lycopene content has shown to be statistically insignificant. However, lycopene levels were different among highly resistant, moderately resistant and susceptible classes. This suggests that there does not exist any correlation between lycopene levels and imparting resistance to plants.

Carotenes are accessory pigments in the light harvesting systems [40]. They are also concerned with photo-protection [41]. Carotene levels calculated for different hybrids shown variable trend. Highest carotene levels were recorded in highly resistant class. However, carotene content in susceptible class was higher than moderately resistant hybrid class. This also suggests that carotene level is not associated with implying resistance to plants.

6. Conclusions

ToMV is considered as a potential threat for tomato cultivation in green/glass-houses because of its contagious nature. In this study we identified tomato hybrids and accessions highly resistant against ToMV using multiple approaches. *Tm2*^{2} gene was successfully transferred to tomato hybrids which was further confirmed using molecular markers. This gene was found highly effective providing complete resistance against ToMV in tomato hybrids. Furthermore, CAPS marker (*NCTm-019)* is validated as efficient and robust marker associated with ToMV resistance in tomatoes. Results of this marker are highly consistent and reproducible with phenotypic data which suggest its potential use in tomato improvement using genome assisted breeding programme. Higher phenolic content, total soluble proteins, better CAT and SOD activities are positively correlated with resistance. On the other side, MDA, photosynthetic pigments (Chl *a* + *b*), lycopene and carotene are pigments essential in light harvesting processes but are not concerned with resistance of plants. The use of identified highly resistant hybrids will be beneficial to manage ToMV problem more efficiently. However, before the use of ToMV-resistant hybrids for general it is very important to check their horticultural characteristics along with market-preferred traits regarding fruit and quality features.

Conflict of Interest

There is no conflict of interest.

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