Indian Journal of Agricultural Sciences **93** (7): 726–731, July 2023/Article
https://doi.org/10.56093/ijas.v93i7.132706

Morphological characterization and molecular validation for ToLCV resistance (*Ty-2* and *Ty-3* genes) in tomato (*Solanum lycopersicum*)

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Received: 28 January 2023; Accepted: 30 June 2023

ABSTRACT

An experiment was conducted during the rainy (*kharif*) season of 2019 and 2020 at ICAR-Indian Agricultural Research Institute, New Delhi to identify the potential genotypes having good horticultural traits along with strong and durable resistance against ToLCV disease of tomato (*Solanum lycopersicum* L.). A diverse set of 30 tomato genotypes carrying different *Ty* genes were screened out at phenotypic and genotypic level. First appearance/symptom of tomato leaf curl virus (ToLCV) disease was recorded in Pusa 120 only after 16 days of transplanting (DAT). Within 30 days of transplanting most of the genotypes, viz. Pusa 120, DT12, DT16, DT6 and Pusa Sheetal recorded 80 to 100 PDI (per cent disease incidence) and they were rated as highly susceptible to ToLCV. While at 90 DAT ToLCV incidence (≤15%) was observed in DT2, DT8, DT17, DT20 and DT30 and were categorized a resistant. None of the genotype was found as highly resistant. Four genotypes, viz. DT2, DT10, DT20 and DT30 showed presence of both resistant genes *Ty-2* and *Ty-3* at genotypic level. These promising genotypes will be useful as parental material to develop lines/hybrids carrying multiple genes against both monopartite and bipartite viruses for strong and durable resistance against ToLCV disease.

Keyword: Screening tomato, Tomato leaf curl virus, *Ty* genes

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetables of family solanaceae grown in India as well as all over the world for both as fresh consumption and processed products, like ketch-up, paste, puree, sauce, chutney, soup etc. It contains lycopene, a red pigment regarded as natural antioxidant. It is a rich source of vitamins especially vitamin A and C, organic acid and dietary fibers and minerals, like iron, phosphorus etc. Due to rich in various health promoting substances, it is sometimes considered as protective foods. Since, it is attractive in appearance and has high nutrient value; it is regarded as poor man's orange in many countries.

The major constraint for tomato growers is the occurrence of tomato leaf curl virus disease in India (Moriones and Navas-Castillo 2000). ToLCV belongs to genus *Begomovirus* of family Geminiviridae, which is transmitted by whitefly (*Bamisia tabaci*). ToLCV disease causes curling, shrinking and cupping of leaves, and leaves become thick rubbery with stunted plant growth, flower is highly affected with few and small flower development (up to 90%) after infection, hence only few and small size fruits are formed which many a time remains immature (Singh *et al.* 2010). Plant breeders are using all possible germplasm from cultivated to wild for identification of resistant source for their utilization in developing line/variety resistant/ tolerant against tomato leaf curl virus.

A thorough understanding about phenotypic and morphological character of any resistance lines of a particular crop carrying specific gene is very important for the production of resistant lines for commercial use. Out of 6 introgressions (*Ty* genes) which confer resistance to tomato leaf curl disease, dominant *Ty-2* and partially dominant *Ty-3* are important for the development of resistant hybrids because of their gene action. Thus, tomato hybrid breeding programmes can be benefited by using tomato lines carrying *Ty-2* and *Ty-3* resistance genes (Prasanna *et al.* 2014). Therefore, the aim of the present work was to assist the disease reaction against ToLCV and validation of *Ty-2* and *Ty-3* genes. Similarly, morphological characterization of resistant lines for horticultural attributes will be helpful for observing implications of level of disease infection and its impact on fruit yield.

MATERIALS AND METHODS

The present experiment was conducted during the rainy (*kharif*) season of 2019 and 2020 at research farm of

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ICAR-Indian Agricultural Research Institute, New Delhi. To evaluate disease resistance and horticultural performance, *Ty* gene introgressed genotypes were transplanted in randomized complete block design (RBD) during rainy (*kharif*) season of 2019 and 2020 (July to November) (Supplementary Table 1). Five plants from each genotype were randomly tagged for disease assessment on the 0 to 4 rating scale described by Banerjee and Kalloo (1987). The disease scoring was done thrice, i.e. at 30, 60 and 90 days after transplanting (DAT). Symptom severity was scored on individual plants according to the following scale:

Upon scoring the genotypes Percent Disease Incidence (PDI) was calculated as:

$$\text{Percent Disease Incidence (PDI)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants examined}} \times 100$$

Upon scoring the genotypes Disease Severity Index (DSI) was calculated as:

$$\text{DSI} = \frac{\text{Total sum of numerical ratings}}{\text{Number of observations} \times \text{Maximum disease rating}} \times 100$$

DNA extraction for the validation of Ty-2 and Ty-3 genes: Young mature leaves (10–15 days) from 30 genotypes were collected and after cleaning with tissue paper kept in polythene bags and finally stored at -80°C for DNA extraction. Genomic DNA of 30 tomato genotypes were isolated with the help of CTAB (cetyl-trimethyl ammonium bromide) method as modified by Murray and Thompson (1980). For the validation of *Ty* genes in different tomato genotypes, 7 different *Ty* genes specific primers, namely JB-1, ACY, FLUW-25, SCAR-1, P6-25 (for *Ty-3* gene) and T0302 and TG105 (for *Ty-2* gene) were used. The amplification was carried out in Ependrof Master Cycler Thermal Cycler with following conditions; initial denaturation at 94ºC for 5 min, denaturation at 94º for 1 min, annealing at 54ºC for 1 min, extension at 72º for 2 min. Amplified product was run in agarose gel along with ladder (50 bp plus, 100 bp, Thermo fisher Scientific) on 2% agarose gel and visualized using UV light. Restricted digestion of 20 µl of amplified *Ty-1* amplicon was performed in a total volume of 20 µl of the *Taq1* enzyme. Scoring of bands was done for each of the gel sections. Allele's size was noted based on the position of bands corresponding to the ladder of known size. The allelic differences in the genotypes were indicated by scoring '+' for the presence of band whereas '-' for the absence of band. If there is heterozygous condition, the scoring was indicated with "+/-" in the data matrix.

RESULTS AND DISCUSSION

Screening of tomato genotypes for resistance to ToLCV under field condition: To tackle the problem of leaf curl disease in tomato, till date, 6 independently inherited tomato leaf curl disease resistance genes and few quantitative trait loci (QTLs) have been mapped in various wild tomato species, namely *S. chilense* carrying *Ty-1, Ty-3, Ty-4* and *Ty-6* genes*, S. habrochaites* syn. *L. hirsutum* carrying *Ty-2*, and *S. peruvianum* carrying *ty-5* gene. *Ty-1* and *Ty-2* genes exhibit complete or nearly complete dominance, while *Ty-3* shows partial dominance. These 3 genes have been extensively used in resistance breeding programs to control both the monopartite and the bipartite *Begomoviruses*. Since the *Ty*-genes in general exhibit partial or incomplete dominance, use of single gene-based resistance has been less effective. Therefore, development of varieties with multiple resistance genes has become imperative for development of tomato genotypes for stable and durable resistance against ToLCV.

In field screening ToLCV disease was recorded only after 16 days of transplanting in genotype Pusa 120. However, after 18 days of transplanting 4 genotypes, namely Pusa Rohini, DT9, Pusa Gaurav and DT16 were found infected with ToLCV (Fig 1). Similar result was found by Govindappa *et al.* (2013). Further, Reddy *et al.* (2010) also reported very high level of disease incidence at early stage of the plants. Thereafter, per cent disease incidence (PDI) of ToLCV was recorded 3 times at 30 days interval. The data of PDI recorded at 60 and 90 days after transplanting (DAT) is presented in Table 1. Within 30 days of transplanting most of the genotypes of tomato were infected by ToLCV. At 90 DAT very low ToLCV incidence (up to 15%) was observed in DT8 (14%) followed by DT2 (15%), DT17 (15%), DT20 (15%) and DT30 (15%), therefore, these genotypes were categorized as resistant (Table 2). The disease severity index (DSI) was also calculated at same interval and it ranged from 0.25 to 1. However, at 90 DAT low DSI (<0.3) was observed in DT2, DT8, DT10, DT17 and DT20.

Coefficient of infection (CI) at 60 and 90 days of transplanting ranged from 2.8 to 100 and 4.5 to 100 respectively. Based on the CI values, all the 30 genotypes were categorized into 5 disease reaction groups, viz. highly resistant (HR), moderately resistant (MR), moderately susceptible (MS), susceptible (S) and highly susceptible (HS). Tomato genotypes were grouped on the basis of their reaction to ToLCV at 90 DAT (Table 2). Out of 30 genotypes, 16 genotypes (Pusa Rohini, Pusa Sadabahar, Pusa120, Pusa Gaurav, Pusa Ruby, DT6, DT9, DT11, DT12, DT13, DT16, Pusa Sheetal, DT21, DT22, DT27 and PKM-1) were categorized in highly susceptible group. Two genotypes, viz. DT25 and DT18 were categorized in susceptible group. Similarly, 2 genotypes, viz. DT23 and DT26 were grouped in moderately susceptible group. Only 4 genotypes i.e. DT24, Kashi Aman, Kashi Chayan and DT7 were categorized in moderately resistant group with CI value 18, 19.2 and 9.9 respectively. Six genotypes (DT2, DT8, DT17, DT10, DT20 and DT30) were categorized in resistant group. None of the genotype showed the disease reaction as highly resistant (Table 2). The photograph of leaves and plant of highly susceptible and highly resistant plant has been given in Fig 1.

It was also noticed that the lines carrying *Ty* genes showed resistance to ToLCV disease especially line having *Ty-2* and *Ty-3* genes together exhibited higher degree of

resistance. Similar, observations were also noticed by Vijeth *et al.* (2018) and Divakaran *et al.* (2008) in tomato lines carrying *Ty* genes. None of the genotype showed the disease reaction as highly resistant. It was also noticed that many genotypes carrying resistant gene showed mild symptom of disease. This might be due to presence of some other viruses which also showed similar symptom in field condition. Therefore, the identification of strain-specific resistant genotypes may benefit regional breeding programmes as region wise virus strain may vary. In the field, many a time mixed infections frequently occur due to transmission vector, as it is possible for whiteflies to transmit more than one types of viruses (Diaz Pendon *et al.* 2010).

On the basis of overall mean value of PDI, DSI and CI, 5 genotypes viz. DT2, DT8, DT10, DT17 and DT20 recorded less than15 PDI and CI values less than 4.5 showing them highly resistant even after 90 days of transplanting.

Molecular screening of tomato genotypes for Ty genes: The availability of PCR based molecular markers for the various resistance loci will significantly reduce the breeding cycle for resistance in tomato and it will simultaneously improve the precise and accurate screening without environment effect. In this direction already several gene specific primers have been mapped by various workers (Hanson *et al.* 2000, Agrama and Scott 2006, Ji *et al.* 2007*)*.

For the validation of *Ty* genes in different tomato genotypes, analysis was done with 7 different *Ty* genes specific primers, namely JB-1, ACY, FLUW-25, SCAR-1, P6-25 (for *Ty-3* gene) and T0302 and TG105 (for *Ty-2* gene).

Primer, FLUW-25 amplified fragments of 475 bp from susceptible lines for *Ty-3* genes, while in resistant lines it showed bands of 641 bp for *Ty-3* gene. Among the 30

Table 1 Reaction of 30 tomato genotypes against ToLCV at 60 and 90 DAT

Genotype	60 DAT				90 DAT			
	PDI	DSI	CI	Reaction	PDI	DSI	CI	Reaction
Pusa Rohini	100	1	100	HS	100	1	100	HS
DT2	10	0.28	2.8	HR	15	0.3	4.5	R
Kashi Aman	28	0.4	11.2	MR	35	0.5	17.5	MR
Pusa Sadabahar	93.9	0.96	90.2	HS	100	1	100	HS
Pusa120	100	1	100	HS	100	1	100	HS
DT6	100	1	100	HS	100	1	100	HS
DT7	10	0.8	8	MR	11	0.9	9.9	MR
DT8	12	0.26	3.12	HR	14	0.29	4.06	R
DT9	60	0.85	51	S	80	0.95	76	HS
DT10	10	0.28	2.8	HR	15	0.3	4.5	R
DT11	25	0.9	22.5	MS	100	1	100	HS
DT12	100	1	100	HS	100	1	100	HS
DT13	70	1	70	HS	94	1	94	HS
Kashi Chayan	24	0.8	19	MR	24	0.8	19.2	MR
Pusa Gaurav	70	0.95	66.5	S	88.4	0.95	84	HS
DT16	100	1	100	HS	100	1	100	HS
DT17	10	0.28	2.8	HR	15	0.30	4.5	R
DT18	20	0.7	14	MR	60	0.8	48	S
Pusa Sheetal	100	0.98	98	HS	100	1	100	HS
DT20	10	0.28	2.8	HR	15	0.3	4.5	R
DT21	100	0.85	85	HS	100	1	100	HS
DT22	90	0.95	85.5	HS	100	1	100	HS
DT23	25	0.8	20	MS	30	0.9	27	MS
DT24	12	0.6	7.2	R	20	0.9	18	MR
DT25	24	0.8	19	MR	52	0.8	41.6	S
DT26	25	0.6	15	MR	34	0.8	27.6	MS
DT27	80	0.85	64	S	100	1	100	HS
PKM-1	70	0.8	56	S	94	0.9	84.6	HS
Pusa Ruby	100	0.95	90	HS	100	1	100	HS
DT30	10	0.28	2.8	HR	15	0.5	7.5	R
CD (P=0.05)	21.0	0.16	7.0		18.0	0.18	8.2	
SEm±	7.3	0.05	2.4		5.1	0.06	2.6	

DAT, days after transplaning.

Table 2 Grouping of tomato genotypes on the basis of their reaction to ToLCV disease at 90 DAT (days after transplanting)

Scale of CI value	Disease reaction	No. of genotypes	Name of genotypes
0–4	HR	0	0
5–9	R	6	DT2, DT8, DT10, DT17, DT20, DT30
10–19	MR	4	DT24, Kashi Chayan, DT7, Kashi Aman
20–39	MS	2	DT23, DT26
40–69	S	2	DT25, DT18
70–100	HS	16	Pusa Rohini, Pusa Sadabahar, Pusa-120, Pusa Gaurav, Pusa Ruby DT6, DT9, DT11, DT12, DT13, DT16, DT21, DT22, DT27, PKM-1, Pusa Sheetal

genotypes, 24 genotypes, viz. DT2, Pusa Rohini, Pusa120, Pusa Sadabahar, DT6, DT8, DT9, DT10, DT11, DT12, DT13, Pusa Gaurav, DT16, DT17, DT18, Pusa Sheetal, DT21, DT22, DT25, DT26, DT27, PKM-1, Pusa Ruby and DT30 were susceptible. The remaining genotypes, namely Kashi Aman, DT7, DT14, DT20, DT23 and DT24 were carrying resistance *Ty-3* genes.

Primer SCAR-1, showed resistance band of 519 bp for *Ty-3* gene, while at 279 bp it exhibited susceptible band. Among the 30 genotypes, 19 genotypes, namely Pusa Rohini, DT2, Pusa-120, DT6, DT9, DT12, DT13, DT11, Pusa Gaurav, DT16, DT17, DT18, DT21, DT22, DT23, DT25, DT27, Pusa Ruby and DT30 showed heterozygous nature and rest genotypes were susceptible. Primer T0305 at 900 bp showed resistant for *Ty-2* gene, while at 800 bp it exhibited susceptible band. Among the 30 genotypes, namely DT2, Kashi Aman, DT7, DT8, DT10, Kashi Chayan, Pusa Sheetal, DT20, DT24, DT26 and DT30 showed resistant for *Ty-3* gene.

Primer-ACY, 132 bp showed presence for *Ty-3* resistance gene in genotypes Kashi Aman, DT7, DT14, DT20, DT23 and DT24 and it amplified fragments of 123 bp in susceptible genotypes, i.e. DT2, Pusa Rohini, Pusa-120, Pusa Sadabahar, DT6, DT8, DT9, DT10, DT11, DT12, DT13, Pusa Gaurav, DT16, DT17, DT18, Pusa Sheetal, DT21, DT22, DT25, DT26, DT27, PKM-1, Pusa Ruby and DT30.

Validation of Ty-3 and Ty-2 genes: *Ty-3* marker was validated with *Ty-3* specific primers like SCAR-1, ACY-1 FLUW-25. Primer SCAR-1 at 519 bp showed resistant for *Ty-3* gene, while at 279 bp, it exhibited susceptible band. *Ty-2* marker was validated with *Ty-2* specific primers like To302 primer. Primer T0302 at bp 900 showed resistant for *Ty-2* gene, while at bp 800 it exhibited susceptible band. Primers like SCAR-1, T0302 and T0305 were also used for validation of *Ty-3 and Ty-2* genes in tomato genotypes (Hussain *et al.* 2019, Lata *et al.* 2019, Mangal *et al.* 2021).

After analysing phenotyping and genotyping data together (Table 3), it was observed that most of the genotypes with the presence of *Ty-3* and *Ty-2* gene showed resistance in the field. Whereas, some exceptional case was found in DT17, Pusa Sheetal and Kashi Aman. DT17 was observed to be resistance up to 90 DAT in the field

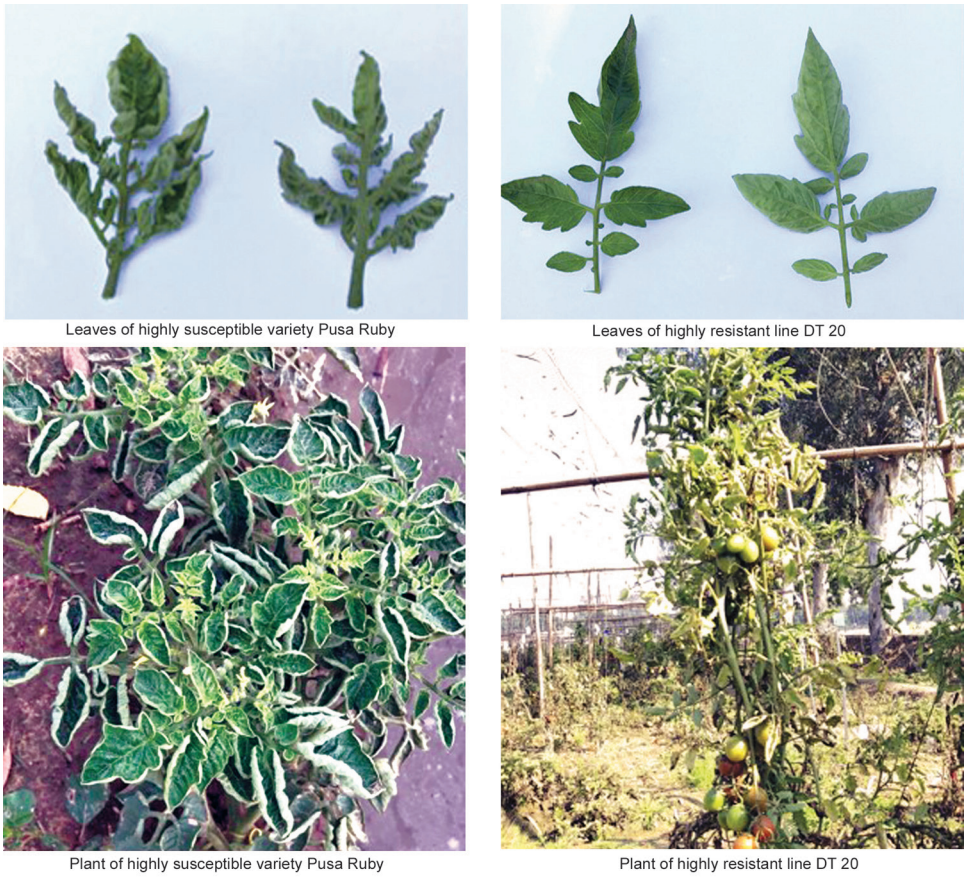

Fig 1 Highly susceptible variety Pusa Ruby and resistant genotypes DT 20 of tomato in the field condition.

Table 3 Comparison of disease reaction (phenotypic and genotypic) with respect to yield in tomato genotypes

Genotype	Field reaction				Yield/plant	*Ty-3*	*Ty-2*
	PDI	DSI	CI	Reaction			
Pusa Rohini	100	1	100	HS	381	S	S
DT2	15	0.3	4.5	R	1470	R	R
Kashi Aman	35	0.5	17.5	MR	776	R	R
Pusa Sadabahar	100	1	100	HS	563	S	S
Pusa120	100	1	100	HS	278	S	S
DT6	100	1	100	HS	230	S	S
DT7	11	0.9	9.9	MR	709	R	R
DT8	14	0.29	4.06	R	1556	R	S
DT9	80	0.95	76	HS	476	S	S
DT10	15	0.3	4.5	R	666	R	R
DT11	100	1	100	HS	262	S	S
DT12	100	1	100	HS	361	S	S
DT13	94	1	94	HS	369	S	S
Kashi Chayan	25	0.8	20	MR	557	R	R
Pusa Gaurav	88.4	0.95	84	HS	332	S	S
DT16	100	1	100	HS	390	S	S
DT17	15	0.30	4.5	R	1050	S	S
DT18	60	0.8	48	S	451	S	S
Pusa Sheetal	100	1	100	HS	372	S	S
DT20	15	0.3	4.5	R	1067	R	R
DT21	100	1	100	HS	160	S	S
DT22	100	1	100	HS	160	S	S
DT23	30	0.9	27	MS	433	S	S
DT24	20	0.9	18	MR	536	R	R
DT25	52	0.8	41.6	S	790	S	S
DT26	34	0.8	27.6	MS	408	R	R
DT27	100	1	100	HS	563	S	S
PKM-1	94	0.9	84.6	HS	286	S	S
Pusa Ruby	100	1	100	HS	400	S	S
DT30	15	0.5	7.5	R	1471	R	R
CD (P=0.05)	18.0	0.18	8.2		68.5		
SEm±	5.1	0.06	2.6		23.5		

condition and yield per plant was also observed to be high but during validation for *Ty-2* and *Ty-3* gene, it was found to be susceptible. Thus, it can be concluded that this genotype may have other source of resistance.

It was also noticed that lines carrying multiple *Ty* resistance genes exhibited strong and high level of resistance, thereby providing resistance to various tomato infecting *begomoviruses*. Prasanna *et al.* (2014) also

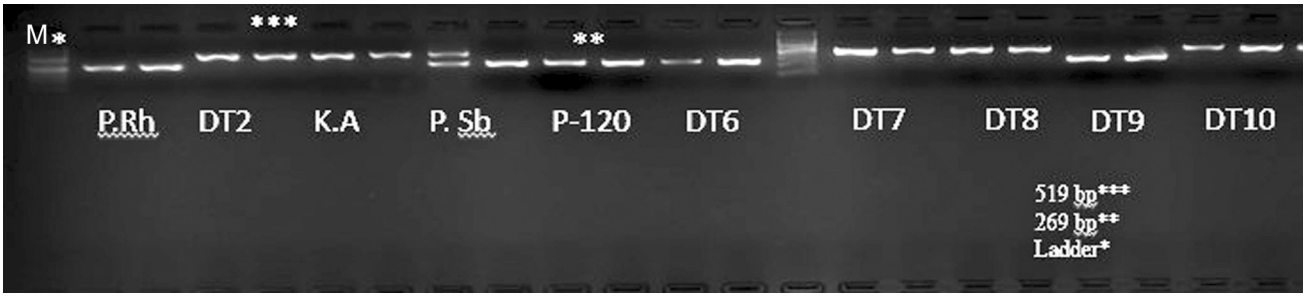

Fig 2 Gel picture showing amplicons obtained with primer SCAR-1.
Each genotypes having 2 replications, Pusa Sadabahar shows admixture in one of the replication showing two bands where another replication represents susceptible band having 269 bp; M-50 bp plus.

found in their study that the pyramided *Ty-2* and *Ty-3* carrying genotypes exhibited high degree of resistance to monopartite as well as bipartite *Begomoviruses*. It was also reported that *Ty-3* is crucial for achieving broad spectrum resistance. The *Ty3* with other genes will be more effective rather than relying on a single gene-based resistance to provide durable resistance due to frequent recombination in *begomoviruses*. Overall this study showed that most of the genotypes with the presence of *Ty-2* and *Ty-3* genes showed resistance in the field. Six genotypes, namely DT2, DT8, DT10, DT17, DT20 and DT30 which were categorized as resistant to ToLCV disease on the basis of phenotypic screening, in the molecular analysis only 4, viz. DT2, DT10, DT20 and DT30 showed presence of both resistant genes *Ty-2* and *Ty-3* at genotypic level. The other 2 genotypes DT8 and DT17 might be carrying some other resistant genes due to which their phenotypic expression was found to be resistant. Therefore, it was further confirmed from this study that for strong and durable resistance against ToLCV disease, there in need to develop lines/hybrids carrying multiple genes for both monopartite and bipartite viruses.

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