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Frequency Incidence of *Tomato Chlorosis* Virus and *Tomato Yellow Leaf Curl* Virus Affecting Tomato Plants

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Abstract: The *Tomato Chlorosis Criniviruses* (ToCV) and *Tomato yellow leaf curl Geminivirus* (TYLCV), which naturally infecting and limited to the phloem, have caused a drastic reduction in tomato yield. The current study aims to determine the incidence of single and mixed viruses using biological, serological and molecular PCR methods in natural tomato plants. The incidence of mixed infection was found more frequently, followed by ToCV and TYLCV (42.3, 28.8, 17.8 in 2020 and 49.1, 29.7 and 19.1% in 2021, respectively). ToCV causes chlorosis, TYLCV causes leaf curl and yellowing, while mixed ToCV & TYLCV cause progress symptoms. By using the heat shock protein 70 (HSP70) and coat protein (CP) genes, ToCV and TYLCV isolates could be identified. These isolates were recorded in GenBank under accession codes "ON951644.1" and "OP265136.1" respectively. Host plants responded differently to severe and common disease density between ToCV and TYLCV. The transmitted whitefly could distinguish between ToCV and TYLCV within 15-20 minutes of the acquisition period. ToCV increases in fields with high whitefly populations, requiring further research to understand effects and reduce harm.

1 Introduction

The tomato (*Solanum Lycopersicum* L.) is one of the most economically beneficial and the largest consumed vegetable crop worldwide, essential for daily consumption and culinary processes. The world produced about 67.5 million tons of fresh tomatoes from 3.7 million hectares (FAO 2020). Several viral diseases cause harm and significantly lower productivity and product quality in tomato plants (Campos et al 2021), depending on the virus species and seasonal growing and region. Whiteflies severely harm and economically devastate susceptible crops (Sani et al 2020) and can cause the dissemination of over 350 virus species in plants, such as Ipomovirus, Crinivirus, Carlavirus, Begomovirus and Torradovirus (Navas-Castillo et al 2011, Luet al 2019, Rodríguez et al 2019). Therefore, B. tabaci is considered a very destructive insect pest glob-ally (Sani et al 2020). The family Closteroviridae of vi- ruses, which includes the genus Crinivirus, is spread *via* whiteflies and has a bipartite genome made up of two separate RNA genomes, independently coated within filamentous virions (Kiss et al 2013). Due to the visible interveinal leaf brittleness and yellowing, which lowers total output, their plant infection can be misinterpreted for nutritional

problems and phytotoxicity (Tzane- takis et al 2013). Tomato chlorosis virus and to- mato infectious chlorosis virus (TICV) account for the most prevalent tomato-infecting criniviruses (ToCV). TYLCV belongs to the family Geminiviridae, genus *Begomovirus* influencing tomato crops globally (Czosnek and Laterrot 1997). TYLCV can be transmitted by a single insect to tomato plants persistently. *B. tabaci* females are better at acquiring and transmitting viruses relative to male insects. Polymerase chain reaction (PCR) offers sensitivity and specificity for the recognition and identification of whitefly-transmitted viruses in infected plants (Mehta et al 1994). Mixed infections with ToCV and TYLCV have become more common and increased within the latest years. The current study aims to examine the incidence of single and mixed ToCV & TYLCV in naturally infected tomato plants and shows how these infections can lead to synergetic disease consequences.

2 Materials and Methods

2.1 Collection of naturally infected tomato plants

Six hundred tomato plants that showed symptoms resembling viral infections were selected and gathered from various governorates during both the 2020 and 2021 seasons. A total of six hundred plants were collected each year from open fields (**Fig 1**). The incidence of tomato viruses was determined based on distanced virus-like symptoms of ToCV and TYLCV.

2.2 Detection of viruses

ToCV, TYLCV, and a mixture of both were detected based on distanced symptoms differential hosts, ELISA, and PCR in eleven selected tomato plants collected from BeniSuef, (Code B) Faiyum, (Code F) Giza, (Code G) Ismailia (Code IS), Monufia (Code M), and Qalyubiyya (Code Q) governorates**.**

2.2.1 ELISA

Two specific polyclonal antibodies specific for both TYLCV provided by Khalid El Dougdoug and ToCV provided by Aly Abdel Salam were used for detecting TYLCV and ToCV infecting tomato plants by enzyme-linked immunosorbent assay (ELISA) according to Clark and Adams (1977).

2.2.2 Differential hosts

Sap inoculation was prepared by extracting naturally infecting tomato plants from Qalyubiyya (Q2), Ismailia (IS1), and Q1 plants with ToCV, TYLCV, or a mixture of both in saline buffer phosphate pH 7.0, 0.1 M. Host plants were inoculated with infectious sap by syringe and kept within greenhouse conditions. The symptoms that emerged were closely monitored daily for 30 days and confirmed by ELISA as illustrated in the results.

2.3 Efficiencies of TYLCV and ToCV Acquisition by Whiteflies

Whiteflies (*Bemsiatabasi*) were obtained from tomato plants that were naturally infected and were identified at the Plant Protection Department, Faculty of Agriculture, Ain Shams University. Non-viruliferous female adult whiteflies were initially positioned in minute (10 cm diameter) self-sealing Petri dishes with moisturized filter paper for the 3-h pre-Acquisition Starvation Period, AAP) and then transferred into tomato plants already infected with TYLCV (code, IS1), ToCV (coded, Q2), or mixture of both (code, Q1) for timed acquisition feeding episodes of 6, 12, 24, or 48 h using 25 insects for each plant. Afterward, virus-carrying insects were moved into uninfected tomato seedlings of the cv. Castle Rock variety, using a 24 h and 48 h AAP and an Inoculation-acquired period (IAP), respectively. The cages were removed, and the remaining whiteflies were killed with a 2% Malathion. The inoculated and non-inoculated plants were cultivated for further six weeks in an insect-proof greenhouse condition to propagate the virus and monitored every day for any external symptoms that may have developed. PCR, RT-PCR, and DAS-ELISA were carried out for the results verification (Li et al 2021).

2.4 Molecular detection for viruses

2.4.1 Extraction of total nucleic acids

Total nucleic acids extraction was carried out from (100 mg) of suspected plant leaves (codes Q2, Q3, and IS1) using a Simply P Virus DNA/RNA Extraction Kit. Positive bulk suspected plants were tested and individually screened using a specific primer for the coat protein (CP) for the Geminiviruses gene and Heatshock-protein70homolog (HSP70h) gene for Criniviruses using polymerase chain reaction (PCR) for TYLCV and Verso ^{TM} one-step RT- PCR kit (Thermo Scientific) for ToCV.

2.4.2 Primer's design

Primers for the Geminivirus CP gene were designed by Accotto et al (2000). Moreover, the heat-shockprotein 70 (HSP70) gene for Criniviruses was designed by Abdel-Salam et al (2019), as shown in **(Table 1).**

2.4.3 Reverse transcription-polymerase chain reaction (One-step RT-PCR)

Total nucleic acids released from the infected plants were employed to amplify the virus's DNA using a real-time PCR reaction with Verso ™ one-step RT- PCR kit (Thermo Scientific). The reaction was accomplished within 25 µl total volume with 4.75 µl of water free from nuclease, 12.5 µl of one-step RT-PCR master mix (2x), 3 µl of total RNA (10 ng /µl), 3 µl of 10 µM of each primer, 1.25 µl RT-Enhancer, and 0.5 µl verso RT-enzyme mix. The process of amplifying was performed in Applied Biosystems ProFlex PCR System While the one-step RT-reaction was initiated with incubation for 15 minutes at 50ºC, then denaturation for 2 minutes at 95ºC. ToCV amplification reaction was acquired in 35 cycles, beginning with 1-minute denaturation at 95ºC, primer annealing (ToCV, 172 +/ ToCV-610 -) 30 seconds at 55°C, followed by 30 seconds of extension at 72°C. At the end of the 35th cycle, the ultimate extension was accomplished at 72ºC for 10 minutes.

2.4.4 Polymerase chain reaction (PCR)

A 2x Taq PCR Master Mix DNA polymerase kit from Biomatik was used to conduct the PCR amplification procedures for TYLCV according to Accotto et al (2000). The PCR products were examined in 1.0% agarose gel electrophoresis, stained with EZ view and examined by a UV illuminator.

2.5 Determination of Pathogenicity

Disease severity values of infected tomato plants with code Q2, single (TYLCV), Q3 single (ToCV), and IS1 double infection in tomato plants were calculated using the following formula (Raupach et al1996). The symptoms index was recorded using the following rating scales: 2 = leaf crinkling & rigidity (LCR); 4 = 50% of leaf curl & narrow (LCN) 6 = 50% of leaf chlorosis (L Ch), = 100% leaf upward cupping and malformation (LUCM) and 10 = venial necrosis, & yellowing (VNY).

$$\text{Disease severity (D.S.)} = \frac{\Sigma\,(\text{disease grade x no. of plants in each grade})}{\text{Total number of plants x highest disease grade}}\ X\ 100$$

3 Results and Discussion

3.1 Single and double infection of TYLCV and ToCV detection

Distanced symptoms of naturally infecting tomato plants infected with either ToCV or, TYLCV, and mixed infection were recorded in the open field during the 2020 and 2021 seasons.

3.1.1 ToCV singly-infected tomato plants (code Q2, Q3)

Early symptoms appear on lower leaves as interveinal chlorosis. The leaves start to be yellow between the veins, and the leaves curl inward slightly. The yellowing regions may also develop a bronzed or brown color, and small, dead spots (flecks) may appear (**Fig 1**). The leaves thicken and the sow tends to be more fragile, breaking easily. The symptoms are predominantly observed on the middle and lower leaves, while the upper leaves seem to be unaffected (Tzanetakis et al 2013).

3.1.2 TYLCV singly-infected tomato plants (code IS1)

Leaf curling and narrow and upper cap shape **(Fig 1).** The leaves become dense and bunched up on the top. The symptoms are primarily recognized on the middle and upper leaves, while the lower leaves seem to be unaffected**.**

3.1.3 TYLCV + ToCV double-infected tomato plants (codes Q1, B, G, M1, F, IS2, M2, M3, IS2)

As symptoms worsen, the yellowing between the veins of the leaves becomes noticeable and leaves curl inward slightly and develop a bronzed color. In addition, necrotic flecking commonly occurs in the yellowing regions **(Fig 1).** Infected plants drastically reduce vigor and fruit yield, as the virus damages the plant's reproductive system and prevents it from producing flowers and fruits**.** These results agree with Abdel-Salam et al (2019), Li et al (2021), and Liao et al (2023). Ac- cording to this study, tomato plants experiencing com-bined TYLCV and ToCV infections displayed a variety of symptoms, including interveinal yellowing and as well as chlorotic and shape distortion of lower leaves. Li et al (2021) found that at 42 dpi, plant visual examinations indicated apparent growth

Table 1. Primers are used to detect Giminiviruses and criniviruses infecting tomato plants

Primers	Nucleotide sequence	Bp	Ref.
Coat Protein gene for TYLCV			
TY1 (+)	5'-GCCCATGTA(T/C) CG(A/G) AAGCC- 3'	580	Accotto et al *2000*
TY2 (–)	5'-GG(A/G) TTAGA(A/G) GCATG(A/C) GTA - 3'		
Heatshockprotein70homologue(HSP70h) gene.			
ToCV, 172 (+)	5'GCT TCC GAA ACT CCG TCT TG 3'	439	Abdel-Salamet al *2019*
ToCV, 610(-)	5' TGT CGA AAG TAC CGC CAC C 3'		

Purine: (A/G), Y: Pyrimidine (C/T), H: (A/C/T), W :(A/T), D: (A/G/T), N: Any nucleotide (A/C/G/T) (Where K = G or T, R = A or G, S = C or G, W = A or T, Y = C or T, B = C, G or T, and V = A, C or G)

Fig 1. Photographs of naturally infected tomato plants showed distanced Chlorosis ToCD assisted Gimini TYLCD diseases symptoms and infested with whiteflies cultivated in the open field.

variations among those that had only one ToCV infection or whose only indication was the presence of chlo- rotic and distorted upper leaves. TYLCV and ToCV double-infected tomatoes exhibited symp- toms, including chlorotic and malformed upperleaves and lower leaves that were chlorotic, wilted,and eventually perished. Ri-beiro et al (2003) and Souza et al (2020) indicated that interveinal chloro- sis, leaf curling, and chlorotic patches are signs of ToCV infection and typically develop first on older leaves in infected plants. These signs and symp- toms resemble those brought on by begomoviruses. Therefore, it is impossible to diagnose be- gomovirus and crinivirus disorders based just on symptoms. In addition, the whitefly *Bemisiatabaci* transmits viruses from both Crinivirus and Be- gomovirus. Tomato severe rugose virus (ToSRV), one of many begomovirus species that have been found in Brazil, appears to be the most common type (Souza et al 2020). ToCV-infected tomato plants display chlorosis between the veins of their lower leaves, which progresses to leaf bronzing and thickening along with the appearance of brown necrotic flecks. Inward leaf curling may also occur on lower leaves. As a result of sterile flowers and deteriorating photosynthesis, symptoms developed in fruits are incoherent and result in a significant drop in production (Tzanetakis et al 2013).

3.2 The incidence of Single and double infection of TYLCV and ToCV

The incidence of naturally infected tomato plants with a single virus (ToCV or TYLCV), and mixed infection were detected during the autumn season of 2020 and 2021 using ELISA in some governorates in Egypt. It was found that the incidence of single infection with

ToCV was 173, 178, TYLCV was 107, 115 and double infection was 254 and 295%. The virus frequency was 28.8, 29.7 (ToCV), 17.8, 19.1 (TYLCV), 42.3 and 49.1 (ToCV + TYLCV) in the autumn season of 2020 and 2021, respectively, out of 600 naturally infected tomato plants **(Tables 2 & 3).** The results indicated that the infection rates were highly infections in 2021 than in 2020 for all single and mixed viruses. In addition, the infection rates were more highly infection in 2021 than in 2020 for all single and double viruses **(Table 2 & 3)** (Esquivel-Fariña et al 2021). Lu et al (2019) evaluated the heights and weights of healthy, single-infected and double-infected tomato plants as well as disease signs and other growth characteristics in tomato seedlings. The governorates differed in the incidence of naturally single and double infections. A high double infection was recorded in Beni Suef and a low double infection rate in Ismailia governorate during the autumn season of 2020, while in the season of 2021, a high double infection was recorded at 54 in Faiyum and a low double infection rate of 35% Monofiya governorate. On the other hand, ToCV caused a high single infection compared to TYLCV during the autumn season of 2020/ 2021**(Tables 2 & 3)** (Kimathi et al 2020). These findings support Lu et al (2019) who reported that the combined infection of TYLCV and ToCV has gradually increased in recent years, and transmission of both viruses is acquired *via B. tabaci* Mediteranean (MED) (Macedo et al 2019). In 2012 and 2013, two chosen tomato fields within the Federal District and Goiás State were surveyed to find out how common criniviruses and begomoviruses were. In total, 150 specimens were gathered and tested using PCR to identify begomovirus and RT-PCR to detect ToCV. Among the examined samples, approximately 48% were found to be infected with both viruses, 20% with the begomovirus ToSRV alone, and 32% with ToCV alone (Lu et al 2019).

Table 2. Incidence of ToCV, TYLCV, and associated (ToCV& TYLCV) in the field at Governorates based on ELISA test cultivated during the autumn season of 2020 & 2021.

Governorates	2020 (n=600)			2021 (n=600)		
	Associated (ToCV & TYLCV)	TYLCV	ToCV	Associated (ToCV & TYLCV)	TYLCV	ToCV
BeniSuef	49	12	25	42	19	22
Faiyum	46	15	27	54	19	27
Giza	42	16	32	53	15	22
Ismailia	30	25	35	37	20	33
Monufia	47	19	29	35	19	40
Qalyubiyya	40	20	25	38	23	34

Table 3. Incidence and Frequency of ToCV, TYLCV, and associated (ToCV & TYLCV) in naturally infected tomato plants in the field

Virus incidence	Naturally infected potato plants			
	2020 Season		2021 Season	
	No	Frequency (%)	No	Frequency (%)
Tomato chlroesis virus (ToCV)	173	28.8	178	29.7
Tomato yellow leaf curl virus (TYLCV)	107	17.8	115	19.1
ToCV +TYLCV	254	42.3	295	49.1

3.3 Differential hosts

Plant host species **(Table 4 & Figs 2 & 3)** belonging to eight families exhibited various reactions at 4-5 leaves–old against ToCV (Q2 isolate), TYLCV (IS1isolate), and ToCV assisted with TYLCV (Q1 isolate). Their reactions were divided into two types. Twenty-three hosts had positive reactions to hosts *with* ToCV and hosts with TYLCV (Kil et al 2015, Abdel-Salam et al2019, Esquivel-Fariña et al 2021). In numerous Mediterranean countries, the symptoms triggered via Tomato chlorosis virus (ToCV) can be easily mistaken for those induced by tomato Infectious Chlorosis Virus (TICV), which is transmitted by white flies. TICV can occur as a single infection or in combination with ToCV in tomatoes (Navas-Castillo et al 2000). When ToCV and TICV coexist in mixed infections without cross-hybridization, it is possible to differentiate between the two viruses through the use of particular antisera, primers, differential hosts, and nucleic acid hybridization (Dovas et al 2002). Additionally, TICV transmission occurred only by *Trialeurodes vaporariorum*, whereas *T. abutilonea*, *T. vaporariorum*, and *Bemisiatabaci* biotypes A and B all can be incorporated in ToCV transmission (Wisler et al 1998). DAS-ELISA testing designated that these samples were positive. Comparable issues were reported by Jacquemond et al (2009) when using DAS-ELISA to identify ToCV. The authors ascribed the difficulties to the TICV low titer in a phloem-inhabiting virus and the heat instability of the studied virions. RT-Plants infected with either ToCV or an unknown TICV were tested using PCR and DAS-ELISA for their abilities to detect and distinguish between the two viruses. Hosts that had previously been tested with immunoassays were retested with RT-PCR using primers designed specifically for ToCV. Natural hosts of ToCV have now been documented in 37 plant species. The current findings also validated Capsicum annuum as previously documented hosts of ToCV (Fortes et al 2012, Mituti et al 2018).

3.4 ToCV, TYLCV singly and double infection

Coded eleven naturally infected tomato plants were selected, which appear to have distinct symptoms of ToCV and TYLCV by ELISA and PCR. ToCV was detected in singly-infected plants in regions of code Q2 and Q3 and TYLCV in nine samples in regions of codes, Q1, B, G, M1, F, IS2, M2, and M3 gave ELISA positive ranging from 0.262 to 0.453, while TYLCV was detected in single plant code IS1 in eight samples codes, Q1, B, G, M1, F, IS2, M2, M3, gave ELISA positive ranging from 0.285 to 0.461 OD at 405 nm. On the other hand, ten ToCV naturally infected tomatoes were detected by RT-PCR and nine TYLCV-infected tomatoes **(Table 5).**

3.5 The DSIs and virus infectivity in tomato plants

The visual observation symptoms expressed differences between single and double infection since they began at 10 dpi in mixed-infected ToCV + TYLCV, while TYLCV and ToCV began at 14 and 16 dpi, respectively, at 50 dpi. The virus infectivity was 85, 90, and 100 percent for double infection and single TYLCV and ToCV infection, respectively. Compared to TYLCV- and ToCV-singly-infected plants, the combination ToCV + TYLCV infected plants' DSI was much higher. The tomato plants that were infected, showed significant chlorosis and leaf wilting. The main sign of ToCV infection in tomato plants was the bottom chlorotic leaves that withered and perished. The primary signs of TYLCV infection in tomato plants were leaf curl and narrow upper leaves, while in ToCV, the main symptom was chlorosis of lower leaves in mixed infected plants that became desiccated and then died **(Table 6 and Fig 4).**

Table 4. The reaction of some host plants inoculated with TYLCV associated with ToCV(Q3 isolate). ToCV (Q2 isolate) and TYLCV (IS1 isolate)

Families	Host plants	ToCV associated with TYLCV(Q1)	ToCV (Q2 isolate)		TYLCV (IS1 isolate)	
		Symptoms	Symptoms	ELISA	Symptoms	ELISA
Amaranthaceae	*Alternantheraret-roflexus*	Ch, LE, LCS, M,	Ch, LE, LCS, Sp	0.294	NS	0.066
	Beta vulgaris L	C, LC, L E,Vch, Y,	C, SP, LE	0.266	LC, LCS, Y,	0.325
	Chenopodium album	B, Ch, LE, mM, Y, VR, NS	B, LE, LCS, mM,	0.301	NS	0.162
	Chenopodi-umamaranticolor	B, LE, LCS, Vch, VC,	B, C, LE, ChS,	0.225	NS	0.162
	Chenopodi-umquinea	LC, M, Vch, VC,	NS	0.071	LC, M, VC,	0.285
Apiaceae	*Ammimajus*	LCh, Y B, C, M, Vch,	LCh, Vch,VY	0.296	NS	0.089
Convolvulaceae	*Ipomeabatatas* (Sweet potato)	B, C, L.E., VY, MCh,	B, C, LCS, mM,	0.271	NS	0.162
Cucrubiacaeae	*Cucumissativus*	Ch, LC, LE, Vch,MY,	Ch, LE, Vch, MY	0.291	LC, L.E., L,	0.362
	Cucurbita pepo	B, LC, Vch,VY, VR	NS	0.071	LC, L.E., LD	0.352
Fabaceae	*Viciafaba L.*	B, C, LE, LCS, Vch,	B,C, LE, Vch, NF, VC,	0.332	NS	0.124
	Phaseolus vul-garis L	B, C, Ch, LE, LCS, VY	Ch, LE, Vch, MY,	0.297	LC, LE, MY	0.382
	Pisumsativum L	B, C, ChM, SP, NF	C, LE, mM, NF	0.332	NS	0.041
	Vignaunguiculata L	B, C, Ch, L.C., L.E., S.P., V.Y	NS	0.071	LC, L.E., LD	0.352
Lamiaceae	*Ocimumbasili-cum*(Basil)	Ch,LC, Vch,VY, ,NF, VR,NS	B,C, LE, mM, Vch, NF, VC,	0.332	NS	0.123
Malvaceae	*Gossypiumbarba-dense*	B, C, C h, LC, V Y, R, S,	Ch, LE, LD, Vch, MY, Y	0.297	LC, LE, MY,	0.382
	Malvapariflora L	B, C, Ch, LC, LD, NF, R, S	C, LE, mM, MCh,	0.332	NS	0.141
Solanaceae	*Daturametel L*	Ch, LC, MMM, M, Vch, MW	Ch, LE, BS Vch,	0.316	LC, LD, LN, ,	0.387
	Daturastramo-nium L.	B, Ch, LE, LD, VY, R, S, VC,	ChS, B, C SP,	0.354	LC, LE, VY,	0.326
	Nicotianaglutinosa.	B, C, Ch, L.C., L.E., L.D, V.Y.,	ChS, B, C, MW	0.341	VN., LC, L.E.,	0.333
	Nicotianarustica L.	C, Ch, LC, M Vch, VY, Y, VR	B, C , Ch ChS, B, C, Ch	0.326	LC, L.E., V.Y., Y, VN	0.323
	*Nicotiana*tabacum *L.*	Ch, LC, L ELD, mM, VY M Ch	Ch, L.E., BS Vch,	0.316	LC, L.D., LCS, L.N., Y,	0.367
	Capsicum an-nuum	B, Ch, L.D., LCS, V.Y., MY, V.C.,	ChS, B, C, Ch	0.354	LC, L.E., V.Y., Y, VN	0.372
	Petunia hyprida	B,C,Ch, ,LE, ,Vch, , NF, Y,	ChS, B,C,Ch	0.321	LC, LE, VY, Y, VN	0.323
	Solanumnigrum	B,C,Ch, ,LE, Vch, MW, VN,	ChS, B,C,Ch	0.329	LC, LE, VY, Y, VN	0.326
	*Solanumtu-berosum.*L	B, ChLE, Vch, Y, VN,	ChS, B,C,Ch	0.332	LC, LE, VY, Y, VN	0.345
	Solanumesculan-tum L.	B, C, Vch,VY, S,VC,Y, VR	Ch, LE, BS Vch,	0.336	LC, LD, LN, Y, S, VY	0.367

- Three replicates for each plant species
- Optical density at 405 nm Negative control= 0.124, Positive control= 0.382
- Symptoms: B: Bronzing, C= crinkling, Ch: chlorosis,ChS: chlorosis spot, L.C.: Leaf curl, L.E.: Leaf epinasty, L.D.: leaf deformation, LCS: leaf cup shape, M: mosaic, mM: mild mosaic, BS: Brown spot, Vch: veinal chlorosis, V.Y.: veinal yellowing, MCh: Marginal chlorosis, M.W.: Marginal waving, MY: Marginal yellowing, NF: Necrotic flecks, NS: No symptoms, R: Reddening, S: Stunting, V.C.: vein clearing, V.N.: vein necrosis, V.R.: Vein reddening, Y: Yellowing
- The sample was considered positive if its OD 405 nm value >2.9 times the healthy tomato control value (O.D405 nm= 0.101). For each sample, results represent the average O.D405 nm of three replicates.

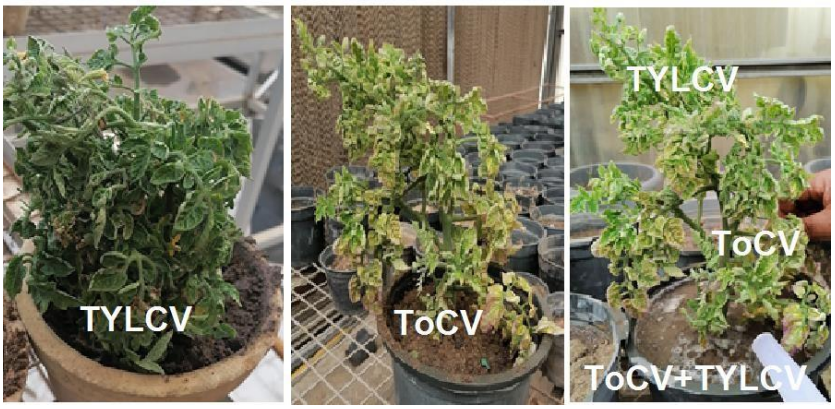

Fig 2. Photograph showing inoculated tomato plants with ToCV associated with TYLCV (Q2 isolate).
ToCV (Q3 isolate) and TYLCV, (IS1 isolate) showed distancing of Chlorosis ToCD assisted Gimini TYLCD diseases symptoms and infested with whiteflies cultivated in the open field.

Fig 3. Host plants for detection of ToCV associated with TYLCV, ToCV, and TYLCV showing different symptoms

Table 5. Detection of ToCV and TYLCV viruses isolates in naturally infected tomato plants in the field exhibit viral symptoms cultivated in the autumn season 2020/2021 in governorate farms

Sample Code& virus-like Symptoms			ToCV		TYLCV	
			ELISA	RT-PCR	ELISA	PCR
Q1		(Ru), (Cu), (L.C), (V.E), (ST), (Ch), (L.N.)	0.432	ve+	0.322	ve+
B		(Ru), (Cu) (ST), (L.C.), (S.T.), (Ch)	0.262-	ve+	0.285	ve+
Q2		(Ch), (Ru), (Ch.B.)	0.453	ve+	0.128	-ve
G		(Ru), (Cu), (Ch), (V.E.), (Ch.B.)	0.293	ve+	0.328	ve+
IS1		(Ru), (Cu), (ST), (LC), (V.E.), (LN)	0.156	-ve	0.461	ve+
M1		(R.u), (C.u), (LC), (UW), (VE), (ST), (Ch.B.), (L.N.)	0.312	ve+	0.306	ve+
F		(Cu), (L.C.), (U.W.), (V.E.), (S.T.), (Ch), (L.N.), (V.Y.)	0.321	ve+	0.295	ve+
IS2		(Cu), (L.C.), (L.N.), (UW), (VE), (S.T.), (Ch), (V.Y.)	0.375	ve+	0.385	ve+

M2		(Ru), (L.C.), (V.Y.), (ST), (ChB), (L.N.)	0.296	ve+	0.359	ve+
M3		(Cu), (L.C.), (VE), (ST), (Ch.B.), (V.Y.)	0.298	ve+	0.305	ve+
Q3		(Ch), (Ru), (Ch.B.)	0.353	ve+	0.153	-ve

- Sample Code, (B) Benisuef, (F) Faiyum, (G) Giza, (IS) Ismailia (M), Monufia and (Q) Qalyubiyya
- ELISA wavelength 405 nm, negative (healthy tomato) control value (O. D_{405nm}=0.124), positiveifits OD_{405nm} value $\geq$2.6times.
- The results represent each sample's average O.D 405 nm of three replicates.
- The Minus value of the $O.D_{405nm}$isbuffer.
- Symptoms: Chlorosis (Ch), Rugosty (Ru), Curling (Cu), Leaf curl (L.C.), Upward cupping (U.W.), Vein enation (VE), Sem twisting (S.T.), chlorosis blotching (ChB), Stunting (St), leaf narrow (L.N.), Vein yellow (V.Y.)

Table 6. Disease severity and Virus concentration of ToCV (Q2 isolate), TYLCV (IS2 isolate), and ToCV associated with TYLCV (Q3 isolate) naturally infected tomato plants

Parameters /Isolates	**Symptoms index (n=20)**						**Virus Infectivity**		
	Symptoms at dpi	**LCR *(2)**	**LCN *(4)**	**LCh * (6)**	**LUCM *(8)**	**VNY (10)**	**% Inf.**	**%DS**	**Conc.**
ToCV (Q2 isolate)	16	2	1	9	5	0	85	0.385	51
TYLCV (IS1 isolate)	14	2	0	1	6	9	90	0.359	74
ToCV + TYLCV (Q1 isolate)	10	0	2	6	4	8	100	Nd	78

TYLCV associated with ToCV (IS2 isolate). ToCV (Q3 isolate) and TYLCV (IS1 isolate).
Total inoculated plants = 20 plant,
* Degree of symptoms index (2), 2 = leaf crinkling & rigidity (LCR), 4 = 50% of leaf curl & narrow (LCN), = 50% of leaf chlorosis (LCh), 8= 100% leaf upward cupping and malformation (LUCM) and 10 = venial necrosis, & yellowing (VNY).
**Virus concentration was determined at the means of three replicates by DAS ELISA (Optical density at 405 nm). Negative control= 0.125

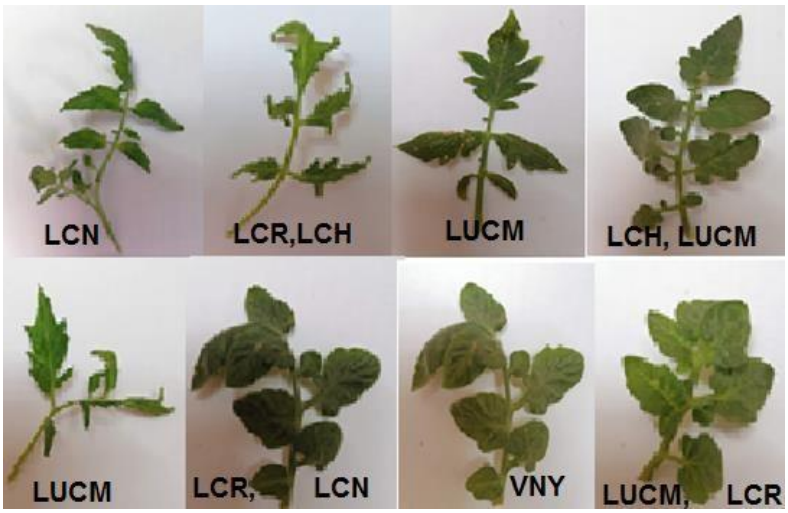

Fig 4. Tomato leaves inoculated with TYLCV associated with ToCV (IS2 isolate). ToCV(Q3 isolate) and TYLCV (IS1 isolate) showing degree ofSymptoms index (2), 2 = leaf crinkling &regusity (LCR); 4 = 50% of leaf curl & narrow(LCN), 6 = 50% of leaf chlorosis (LCh), 8 = 100% leaf upward cupping and malformation (LUCM) and 10 = venial necrosis, & yellowing (VNY)

3.6 Whitefly transmission efficiency

Twenty whiteflies/tomato plants efficiently transmitted TYLCV & ToCV by applying for 4 hr. of AAP at 45% and IAP at 45% for 2 hr. The maximum transmission efficiency (90 and 100%) was achieved by applying AAP and IAP, respectively by apply for 48h **(Table 7).** The whitefly transmitted showed the ability to distinguish between ToCV and TYLCV in mixing them through the period of its acquisition of the two viruses, whereas, ToCV was transmitted after two hours, and TYLCV was transmitted after eight hours during the feeding period **(Table 7).** This finding is very different from the situation with TYLCV, which is a transmissible, proliferative virus (Pan et al 2012). A recent study revealed that ToCV cannot be identified in the adults of the first generation by vertical transmission, whereas TYLCV can be recognized in first-generation nymphs and eggs but not pupae and adults (Pan et al 2012). Furthermore, Wei et al (2017) reported that TYLCV entry into the reproductive organ of its vector primarily depends upon the age of the adult and can be retained in two generations at minimum in the lack of virus-infected plants, and considered ToCV as a semi-persistent virus. Re- garding the inoculation and acquisition of ToCV via *B. tabaci* MED whiteflies, we found no differ- ences in the acquisition effectiveness among fe- male and male whiteflies, signifying no association between the acquisition efficiency of ToCV and gender. As they could exhibit significant epidemiological and ecologic repercussions, the im- pacts of synergetic interactions during combined viral infections on how the vector acquires and transmit vi- ruses warrant specific investigation. Higher vector transmission may be a consequence of the higher con- centration of viruses in combined infections (Froissart et al 2010). For instance, ToCV and TICV transmission efficiency of whiteflies matched concentrations of virus accumulated in hosts during combined and single infec- tions (Wintermantel et al 2008). Additionally, syner- getic interactions that make the viruses more patho- genic can worsen plant damage, particularly in cultivars that are vulnerable to it (Murphy and Bowen 2006, Tatineni et al 2010). In this work, plants infected with ToCV+TYLCV together produced disease synergism. There are many TYLCV-resistant cultivars available, but their resistance to ToCV needs to be tested. Although no tomato cultivar has shown resistance toward both TYLCV and ToCV, breeding for resistance to these viruses may be an efficient way to manage such synergetic disease.

3.7 Molecular characters of ToCV and TYLCV

3.7.1 PCR amplification

The amount and integrity of the nucleic acid from infected tomato leaves were confirmed by Nanodrop at A260/280 absorbance ratio was 1.8, designating improved yield and high purification. The ToCV /cDNA using one-step RT-PCR amplified a fragment

Table 7. Whiteflies transmission efficiency of TYLCV associated with ToCV (Q3 isolate) on tomato plants

Time	Acquisition access period (AAP)				Inoculation-acquired period (IAP)			
	No. of infected plants (n=20)	Transmission			No. of infected plants (n=20)	Transmission		
		**%	ToCV	TYLCV		**%	ToCV	TYLCV
10 min	0	0	ND	ND	0 / 20	0	ND	ND
20 min	0	0	ND	ND	0 / 20	0	ND	ND
40min	0	0	ND	ND	2 / 20	0	ND	ND
60 min	0	0	ND	ND	5 / 20	0	ND	ND
2 hr	0	0	ND	ND	9 / 20	45	+	ND
4 hr	9	45	ND	ND	13 / 20	55	+	ND
8 hr	12	60	+	ND	15 / 20	65	+	+
16 hr	15	75	+	+	18 / 20	90	+	+
32 hr	20	100	+	+	20 / 20	100	+	+

- Twenty-five whiteflies /plant
- No. of infected / No. of an inoculated plant **(n=20)**
- **% Transmission = No. of infected / No. of inoculated plant × 100 was confirmed by ELISA &PCR

of around 438 bp, which corresponds to the C-terminal region of the HP70 gene **(Fig 5).** The TYLCV /DNA using PCR amplified a fragment of about 550 bp specific primers **(Fig 5).** Therefore, general primers and nested-PCR techniques targeted HSP70 Criniviruses and TYLCV (Li et al 2021). When there is a mixed infection, the two viruses can be distinguished using certain antisera, particular primers, distinct hosts, and hybridization of nucleic acid in cases when both viruses do not express cross-hybridization (Louro et al 2000).

3.7.2 Nucleotide sequence

The partial nucleotide sequence of the HSP70 gene (ToCV) fragment amplified by PCR was recorded as ON951644.1 in GenBank and the specific CP gene of TYLCV isolate was recorded as OP265136.1 in GenBank. In addition, the partial nucleotide sequence of the PCR-amplified was done to compare it to other endorsed ToCV& TYLCV isolates recorded in GenBank. The sequencing has been accomplished from the forward direction at Macrogen, Korea **(Figs 6 & 7).** Moreover, investigations on the diversity of ToCV (Orfanidou et al 2014) and TYLCV indicated that its lowered evolution rate may be related to the increased negative selective pressure, which enables the virus's quick spreading through tomato-producing regions (Li et al 2021).

3.7.3 Viro-informatic Analysis of molecular data

The phylogenetic tree of ToCV Egyptian isolate revealed limited genetic variability with recoded available in NCBI GenBank different host species **(Fig 6).** From the results, we found 4 clusters cluster 1 included China Shandong KY679890.1, China Liaoning Liaozhong MF278017.1, Italy Campania with 100%, cluster 2 included Pakistan MN869006.1 99%, cluster 3 included Japan LC528263.1, south Korea MG813911.1, with 100% and cluster 4 included Nobaria isolates MK161109.1 with 98 %

Phylogenetic analysis and nucleotide sequence of TYLCV Egyptian isolates were subjected to purification and sequencing. The partial CP gene of the Ismailia isolate was resolved and registered in the NCBI-GenBank with the accession number: OP265136.1. The phylogenetic analysis indicated restricted genetic variation within tomato TYLCV isolates from Egypt and those of other isolates registered in the NCBI, regardless of the host plant species from which they were isolated **(Fig 7).** The tree included 2 clusters, including Turkey Mugla AJ867487.1, Tunisia KU958498.1 South Korea MK521830.1 and China ON093146.1 with 99 %, and Cluster 2 included Spain Murcia DQ058102.1, Jordan GQ861426.1, and Egypt Ismailia MT890681. with 98 %. On the other hand, 2 clusters were similar with local isolate 95%.

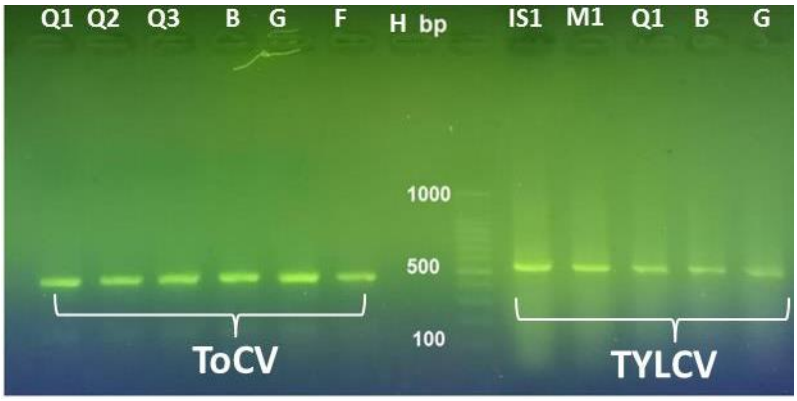

Fig 5. Agarose 1.5% Gel electrograph showing PCR products expected size 439 bp for Heat shock protein 70 homologue (HSP70h) gene / ToCV and CP gene-specific primers 580 bp for Geminivirus / TYLCV detected naturally infected tomato plants. Sample Code: (B) Benisuef, (F) Faiyum (G) Giza (IS1, IS2) Ismailia (M1, M2, M3) Monofiy and (Q1, Q2, Q3) Qalyubiyya, Line (7) Healthy (negative control) and Leader (L) 100 bp

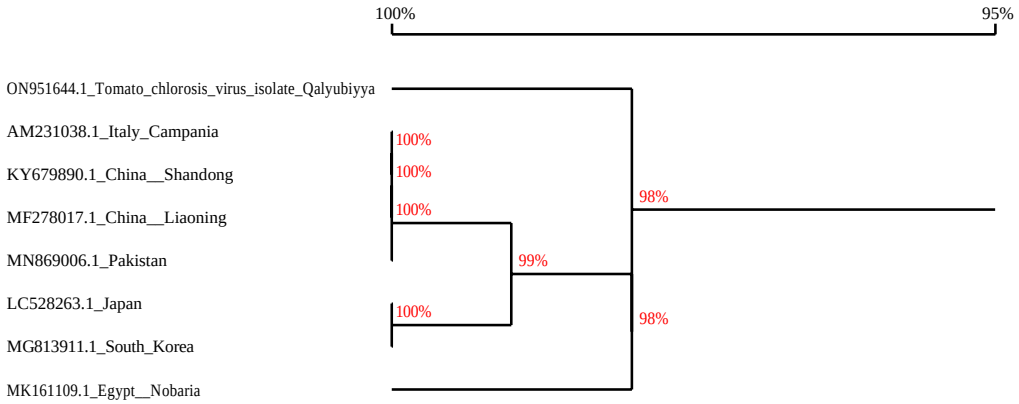

Fig 6. A phylogenetic tree based on partial nucleotide sequences of the HSP70h gene obtained from 8 ToCV isolates. ON951644.1 virus isolates were obtained from the present study, while other isolates were retrieved from NCBI- Gen-Bank.

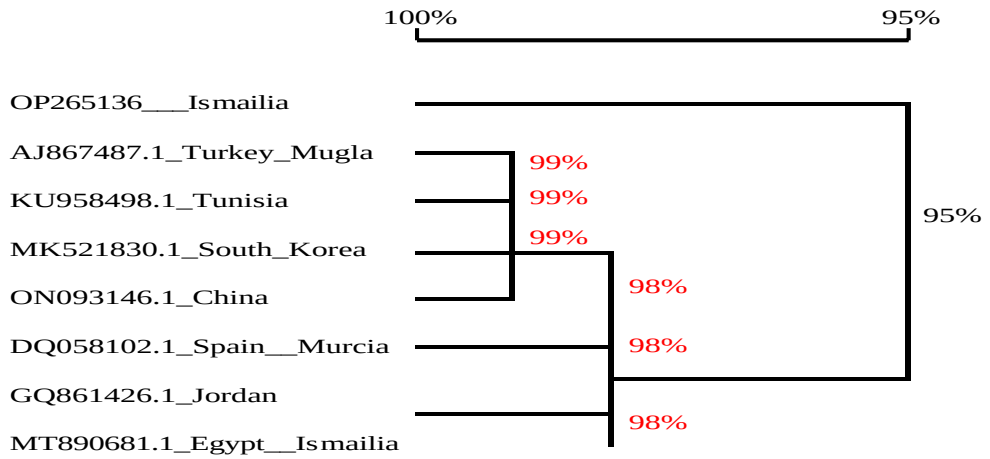

Fig 7. A phylogenetic tree based on partial nucleotide sequences of the specific gene obtained from 8 TYLCV isolates. OP265136.1 virus isolates were obtained from the present study, while other isolates were retrieved from NCBI-GenBank.

In this study, synergistic interactions between ToCV and TYLCV can increase plant damage and yield losses, so breeding for resistance cultivars can potentially help control such synergetic disease (Murphy and Bowen 2006, Tatineni et al 2010).

4 Conclusion

The incidence of Tomato yellow leaf curl Geminivirus associated with Tomato chlorosis Criniviruses infecting tomato plants increased with high whitefly populations in the field. The increased prevalence of ToCV and TYLCV in fields having increased whitefly populations underscores the necessity to investigate this virus in order to better comprehend its impact on tomato crops and reduce any possible damage.

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