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Research Article
Evidence for Upholding the Association of Whitefly Species *Bemisia tabaci* and *Trialeurodes vaporariorum* with *Tomato Yellow Leaf Curl Virus*

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Abstract

Background and Objective: *Tomato yellow leaf curl virus*, a member of the genus *Geminivirus* is known for a steep decline in tomato yield recorded during the past two decades around the globe and for influencing the horticulture industry. *Bemisia tabaci* (silver leaf whitefly) is known to be the major insect vector transmitting TYLCV. Apart from it, the greenhouse whitefly (*Trialeurodes vaporariorum*) has also been encountered feeding and rearing on tomato plants in cool temperate regions of Himachal Pradesh raising doubts about the spread of TYLCV via these vectors in these areas. The present investigations were therefore carried out to understand the virus vector relationship between the two whitefly species of the warmer and colder regions with TYLCV on tomatoes using serological and molecular tools. **Materials and Methods:** One hundred leaf samples and 3-5 whitefly species were randomly collected from 50 locations in three Districts Una, Solan and Sirmour of Himachal Pradesh and tested using DAS-ELISA against TYLCV antisera. Positive culture of TYLCV was maintained and used to test the transmission efficacy of whitefly vectors over varying inoculation access periods and whitefly vectors were also characterized on a molecular basis using polymerase chain reaction. **Results:** Disease incidence ranged between 2.5-90% and pest incidence from 10-80% in the case of each whitefly sp. and TYLCV was detected in most of the plants tested whereas *B. tabaci* recorded the highest concentration of TYLCV in comparison to *T. vaporariorum*. Transmission efficacy also remained highest for *B. tabaci* within the inoculation access period of 4 hrs and *T. vaporariorum* failed to transmit TYLCV even after 24 hrs of feeding. The PCR also successfully characterized these two whitefly vectors proving them to be different not only on morphological characters but on a molecular basis as well. **Conclusion:** The TYLCV has been found prominent in warmer areas and actively spread by *B. tabaci* posing a serious concern for farmers *T. vaporariorum* remains a non-concerned pest for the farmers.

Key words: *Tomato yellow leaf curl virus*, *Bemisia tabaci*, *Trialeurodes vaporariorum*, *Geminivirus*, DAS-ELISA, Polymerase Chain Reaction (PCR), virus-vector relationship

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tomato (*Solanum lycopersicum*), a Solanaceous crop is cultivated for its edible fruit largely in outdoor fields, greenhouses and net houses by both small scale farmers as well as commercial growers throughout the world for consumption and has ample nutrition (vitamin C, phenolics, flavonols and anthocyanins) and antioxidant properties (tocopherols, lycopene and β-carotene)[1-3]. Tomato cultivation besides culinary purposes is also gaining popularity due to its excessive use in numerous processing industries to prepare sauce, puree, soup, ketchup and juice for retailing at a large scale in many trading markets[4]. Hence, a healthy and high-yielding crop becomes the primary demand for the growers to gain profits on the economic front.

In India, agricultural parameters such as temperature, soil, humidity etc favour the cultivation of tomatoes at a high rate. However, pests like fruit borers (*Helicoverpa* and *Spodoptera*) also sap suckers (whiteflies, jassids and leaf miners) and diseases caused by bacteria (bacterial spot), fungi (*Fusarium wilt*) and viruses (*Tomato yellow leaf curl virus, Tobacco mosaic virus and Cucumber mosaic virus*) play a key role in retarding the yield causing financial harms to the producers[5-6]. Therefore, the present scenario's utmost challenge for tomato growers in India is to control the population of whiteflies and manage the spread of the *tomato yellow leaf curl virus* through these vectors.

Whiteflies were earlier detected in tobacco and were named *Aleyrodes tabaci* and *Bemisia tabaci* in Greece in 1889[7]. According to their feeding tendency, they are characterized among the most dangerous pests of tropical and subtropical areas and are found extracting sap from several agricultural, horticultural and various weeds present in the surroundings of these commercial crops. These whiteflies not only weaken the plant but also expose them to other major fungal and bacterial diseases[8]. Besides subduing the plant immune response they play a vital role as carriers of *tomato yellow leaf curl virus* transmitting it from one plant to the other in a persistent circulative manner[9].

Tomato yellow leaf curl virus (TYLCV) is a dsDNA molecule from the genus *Begomovirus* and family *Geminiviridae*, considered one of the most devastating of all viruses infecting tomatoes causing a huge drop in its yield up to 100%[10-12] thereby causing a significant economic downturn for the tomato growers. TYLCV is transmitted only via whitefly *B. tabaci* and other modes like seed, dodder, pollen, mechanical etc., have not been reported anywhere from India or any other country around the globe responsible for its spread[9,13,14].

For a better understanding between the virus and the associated vector, serological assay mainly DAS-ELISA is used which not only helps in detecting the virus in symptomatic plants but also helps in determining the virus particles in the gut of those vectors transmitting them in a circulative persistent manner[15,16]. However, molecular assays like PCR help in characterizing the exact identity of the insect vectors like whitefly which are very small to identify in the techniques of the field like PCR are widely used[17,18].

In Himachal Pradesh, both *B. tabaci* and *T. vaporariorum* are prevalent in different climatic conditions suitable for their survival as *B tabaci* prefers warmer areas having less wind and on the contrary *T. vaporariorum* enjoys reproducing in colder regions.

The present investigations were therefore carried out in the direction of uncovering virus vector association between TYLCV and two whitefly species by recording the occurrence and prevalence of TYLCV in tomato fields and unravelling its transmission behaviour from plant to plant that can help the tomato growers in the state to retain their economy.

MATERIALS AND METHODS

Study area: Field surveys were conducted between 2019-2021 at 115 major tomato-growing locations in the Una, Solan and Sirmour Districts of Himachal Pradesh.

Surveys: Intensive surveys were conducted in tomato fields to collect data for the presence of TYLCV based on visual symptoms and witness the associated whitefly vector on them. A 'W' shaped pattern was followed to screen the fields and plants were marked accordingly, symptomatic two to three leaves were collected from each plant in separate polythene bags. Later, whiteflies found on symptomatic plants were collected using mouth aspirators and were identified on morphological characters. All the samples bearing symptomatic leaves and whiteflies were brought to the laboratory in an icebox for carrying out experimental detection assays. A few symptomatic plants were also brought from the field and maintained in the glasshouse to carry out transmission studies. The percent disease incidence and percent insect incidence were calculated by using the following formula[18,19]:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

$$\text{Insect pest incidence (\%)} = \frac{\text{Number of insects on plants}}{\text{Total number of plants observed}} \times 100$$

Serological detection: Alkaline Phosphatase (ALP) based double antibody sandwich ELISA (DAS-ELISA) commercial kits from BIOREBA AG, Switzerland were used for serological detection of TYLCV in all the 230 collected samples of tomato (2 from every 115 fields) and whiteflies (5-10 collected from random 10 plants) as per the instructions of the manufacturer. All the samples were crushed in an extraction buffer and collected sap was used in the assay. The absorbance value for each sample was read at 405 nm to record the O.D. using iMark Microplate Absorbance Reader (Bio-Rad, USA). Microtiter plates were kept in dark at room temperature in a humid box for 15-60 min till the development of yellow colour. The reaction was stopped by adding 50 µL of 3M NaOH to each well if desired. The results obtained in ELISA tests were interpreted and the samples with O.D. values twice the mean values of healthy control samples were considered to be carrying *tomato yellow leaf curl virus* [20].

Molecular detection: Whiteflies collected from tomato fields based on morphological traits were further characterized at the molecular level to know their exact identity[21].

DNA extraction from whiteflies: Total genomic DNA from each whitefly specie was extracted by grounding it to a fine paste using a micro pestle and 700 µL of pre-warmed CTAB extraction buffer was added to each tube. All tubes were then incubated at 65°C for 1 hrs in a Shaking Water Bath (nuveST30, Turkey) and later each tube was filled with an equal volume of 700 µL of chloroform: isoamyl alcohol (24:1). The contents were mixed thoroughly and the tubes were spun at 12,000 rpm for 12 min. (Eppendorf, 5430 R) at 25°C. The aqueous phase was transferred to new tubes and 450 µL prechilled isopropanol was added and kept at -20°C for 1 hr to precipitate the DNA. Then the tubes were spun at 10,000 rpm for 12 min and the supernatant was decanted. The DNA pellet was washed thrice with 70% ethanol, dried and dissolved in 100 µL of Tris EDTA (10 mM Tris-HCl and 1mM EDTA pH 8.0)[22]. Isolated DNA was quantified using UV BioSpectrophotometer (Eppendorf, Germany) at 260/280 nm and stored at -20°C for further use.

PCR amplification: PCR amplification was carried out using 1X PCR pre-mix procured from Genet Bio, Korea using Mitochondrial cytochrome oxidase subunit I gene (mtCOI gene) primers[23], mtCOI (forward) 5' GGTCAACAAATCA TAAAGATATTGG 3' and mtCOI (reverse) 5' TAAACTTCAGGCTG ACCAAAAAATCA 3'. A total volume of 20 µL reaction mixture (10 µL of Taq Buffer+MgCl2+dNTPs+Taq DNA polymerase, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of template DNA and 6 µL nuclease free water) was prepared and vortexed for PCR run with conditions (Initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and final elongation at 72°C for 10 min)[23]. Amplified products were visualized in 1 % agarose gel using a Gel Documentation System (BioRad, USA).

Virus vector relationship: Glasshouse studies were carried out for a better understanding of the transmission efficiency of whitefly species *B. tabaci* and *T. vaporariorum* from TYLCV-infected tomato plants to healthy tomato plants.

Maintenance of whitefly colonies: Host plants like sunflower, cotton, brinjal, chilli, tomato and okra were raised in pots filled with soil+compost mixture having (2:1) proportion in a controlled growth room and whiteflies collected from the fields were released on these seedlings at two to the four-leaf stage for multiplication provided with temperature was 28°C, relative humidity 30-50% and photoperiod of 14 hrs[24]. During the process of colony maintenance, older seedlings were replaced by new seedlings for better feeding and reproduction of whiteflies in each host and whiteflies on each host were reared successfully for two generations to get non-viruliferous (healthy) flies.

Transmission studies: Different-sized plastic tubes were used to form cages and their bases were removed using a soldering rod and then fixed with a muslin[25] cloth, then a small hole (0.5 cm) was created in the middle of these tubes to facilitate the release of whiteflies on infected tomato plants. Symptomatic plant leaves were inserted into the tube where one end of the tube was covered using a cotton plug then *B. tabaci* and *T. vaporariorum* both were released separately into the cages through a small hole. After a 24 hrs acquisition access period about 300-500 adult viruliferous whiteflies were then collected and transferred to healthy tomato seedlings and allowed to feed with different inoculation access periods ranging from 4-24 hrs. After inoculation, the whiteflies were removed and 1.5% Hostathion (Traizophos) spray was used on seedlings to kill the whiteflies. Inoculated plants were observed for 12-14 days as virus symptoms usually appeared two weeks after inoculation.

DAS ELISA detection of TYLCV: The presence of TYLCV was confirmed on the tested plants based on expressed symptoms after 2 weeks of probing by viruliferous whiteflies and was

reconfirmed with a DAS-ELISA assay. Leaf samples from both *Bemisia tabaci* and *Trialeurodes vaporariorum* infected plants were tested and O.D. at A_{406} nm was recorded.

Statistical analysis: The study obtained an O.D. value $\geq 2\times$ the O.D. value of negative control (All O.D. values will be considered positive for the virus).

RESULTS

Percent incidence of TYLCV and whitefly species: Surveys were conducted between 2019-2021 for recording the occurrence and distribution of TYLCV and encountering whitefly species on tomato plants at 115 major tomato growing areas in the Una, Solan and Sirmour Districts of Himachal Pradesh. Percent disease incidence of TYLCV was based on the symptoms recorded in the surveyed fields in the form of mosaic, mottle, yellowing, leaf deformation, cupping, puckering, curling, dwarfing and shoe stringing. However, whitefly species were first distinguished on their morphological traits and then percent incidence was recorded according to their number on each plant marked.

Tomato yellow leaf curl virus was found to prevail in almost all 115 locations surveyed based on the symptoms expressed by tomato plants and the maximum incidence of TYLCV was recorded at Charhatgarh (95%) followed by Fatehpur (92%) whereas the minimum incidence was recorded at Ajauli (41%) in district Una. In Solan District, the maximum incidence was at Sai (76%) followed by Baddi (75%) and the minimum incidence at Shili (9.0%) was recorded whereas, in District Sirmour, the maximum incidence was recorded at Renuka (46%) followed by Nahan (33.5%) and minimum at Kuftu (0.7%) (Table 1).

Whitefly species *Bemisia tabaci* was found prevalent in hot areas identified as big-sized with round wings and yellow bottom whereas *Trialeurodes vaporariorum* was observed at a higher rate in the colder region identified as small in size with triangular wings (Fig. 1a-c). Based on such characteristics data on occurrence was collected and *B. tabaci* with maximum occurrence was recorded in Charhatgarh (65.7%) followed by Fatehpur (64.1%) and minimum occurrence in Rampur Bela (12%) in district Una. In Solan District, the maximum occurrence of *B. tabaci* was recorded at Sai (80.5%) followed by Baddi (64.5%) and the minimum occurrence at Halda (9.0%) and percent occurrence of *Trialeurodes vaporariorum* was recorded as a maximum at Pabyana (62%) followed by Byas (56%) and minimum occurrence of 0.2 % in Nehar Pab in District Sirmaour (Table 1).

Serological-based (DAS-ELISA) assay for TYLCV in tomato plants and whiteflies: Marked symptomatic plants were subjected to DAS-ELISA for detecting the virus using leaves as explant and therefore, O.D. values obtained after the test confirmed the presence of TYLCV in all three districts (Fig. 2).

Fig. 1(a-c): *Bemisia tabaci* and *Trialeurodes vaporariorum* collected in aspirator from plants, (a) *Bemisia tabaci* present on wild herbs grown in the tomato fields, (b) *Trialeurodes vaporariorum* present on wild herbs grown in the tomato fields and (c) Whiteflies collected in aspirator from different tomato fields to carry out further studies

Table 1: Incidence of *tomato yellow leaf curl virus* and whiteflies on tomato plants

Serial number	Locations	Disease incidence (%)	Insect pest incidence (%)
UNA			
1	Nangal	82.0	50.0
2	Mehatpur	78.0	64.1
3	Anandpur Sahib	61.0	46.5
4	Basal	53.0	22.0
5	Chalola	76.5	36.0
6	Gagret	81.0	29.0
7	Amb	80.0	42.5
8	Nandpur	72.0	50.0
9	Baduh	41.5	15.0
10	Basoli	58.0	30.0
11	Tabba	55.0	50.6
12	Gugaroo	61.0	43.0
13	Chintpurni	54.5	26.3
14	Bharwain	52.0	25.0
15	Rajpura	86.0	31.5
16	Haroli	91.0	75.5
17	Basrara	64.0	18.0
18	Charhatgarh	95.0	65.7
19	Fatehpur	92.0	64.1
20	Daulatpur	56.0	24.5
21	Ambota	60.0	32.0
22	Ajnoli	72.0	36.6
23	Batuhi	78.0	41.0
24	Bhadsali	65.5	30.1
25	Ajauli	41.0	26.0
26	BanGrh	89.0	40.5
27	Dangoli	46.0	15.4
28	Galua	53.0	27.5
29	Fatehwal	80.0	16.7
30	Chatra Khas	76.0	60.0
31	Jhalera	66.5	45.0
32	Kasba	62.0	27.5
33	Majara	53.0	40.0
34	Nagnuli Har	58.5	20.0
35	Salangari	60.0	15.2
36	Rampur Bela	42.0	12.0
37	Sanjhot	86.0	80.1
38	Udheypur	50.0	33.5
39	Surjehra	42.0	15.0
40	Sasan	69.0	30.0
SOLAN			
1	Jatoli	28.0	20.3
2	Majhgaon	40.0	50.0
3	Shamror	36.0	32.0
4	Dhilon	42.5	18.5
5	Deothal	40.0	20.0
6	Nauni	19.0	15.1
7	Albora	22.0	20.0
8	Bagor	36.0	32.5
9	Anun	24.5	16.0
10	Barog	20.0	12.0
11	Sai	76.0	80.5
12	Baddi	75.0	64.5
13	Nalagarh	61.0	29.0
14	Deothi	41.0	15.7
15	Sabathu	38.0	20.6
16	Chapla	20.5	12.0
17	Chilri	32.0	36.0
18	Kalaghat	26.0	10.5
19	Shamti	25.0	20.0
20	Haripur	30.0	15.2

Table 1: Continue

Serial numbers	Locations	Disease incidence (%)	Insect pest incidence (%)
21	Jarai	18.0	10.1
22	Dharampur	11.0	10.5
23	Arki	43.5	31.5
24	Badkhor	39.0	22.0
25	Charjera	26.0	11.3
26	Galanag	31.0	40.1
27	Tiwakri	25.5	42.0
28	Thapo	18.0	16.4
29	Shili	9.0	10.0
30	Sehal	11.0	10.0
31	Lakharanji	25.0	12.5
32	Mahlog	21.0	20.0
33	Khalwa	30.0	20.5
34	Halda	36.0	9.0
35	Ghori	20.5	18.8
36	Manjhar	17.0	20.7
37	Nehr	19.0	30.0
38	Ranga	46.0	45.0
39	Tikar	32.0	17.0
40	Bhoj Nagar	38.5	25.5
SIRMOUR			
1	Batol	15.0	26.0
2	Dahan	18.0	17.3
3	Jola	21.5	10.0
4	Darena	6.0	2.4
5	Dhamandar	18.0	13.0
6	Tikri Jijah	25.0	10.5
7	Palu	3.0	4.0
8	Karoli	18.0	10.0
9	Kulath	20.0	32.0
10	Rajgarh	25.0	20.1
11	Nahan	33.5	20.0
12	Renuka	46.0	18.2
13	Chhog Tali	29.0	21.5
14	Dhamla	21.0	10.0
15	Lanaru	4.0	1.6
16	Nehar Pab	3.5	0.2
17	Phagu	10.0	15.0
18	Rihana	15.0	6.0
19	Sanora	26.0	31.0
20	Pabyana	28.0	62.0
21	Bhutli	16.0	40.5
22	Gawahi	13.0	15.0
23	Dharja	18.0	19.0
24	Giripul	26.5	32.0
25	Sail	11.0	4.6
26	Badiana	23.0	10.0
27	Anji	16.0	24.0
28	Ser	12.5	13.1
29	Kuftu	0.7	0.6
30	Bhalana	10.5	22.0
31	Tharu	21.0	36.0
32	Rana Ghat	19.0	10.4
33	Amboa	20.0	35.0
34	Byas	24.0	56.0
35	Chandol	14.0	27.2

%: Percent

Maximum O.D. value of 1.210 was recorded from a plant expressing puckering, mosaic and dwarfing symptoms collected from District Una followed by an O.D. of 0.724 of a plant expressing mosaic collected from District Solan and an O.D. value of 0.466 of a plant with mosaic symptoms collected from District Sirmour as shown in (Table 2).

Table 2: Serological assay of TYLCV using DAS-ELISA

Serial numbers	Symptoms	O.D. A_{405} nm
UNA		
1	Mosaic, curling, leaf deformation	0.743 (+)
2	Yellowing, cupping	0.689 (+)
3	Yellowing, leaf deformation	0.116 (-)
4	Dwarfing, mosaic	0.201 (-)
5	Shoe stringing, yellowing	0.701 (+)
6	Mosaic, cupping	0.724 (+)
7	Mottle, leaf deformation	0.662 (+)
8	Shoe stringing, mosaic, leaf deformation	0.686 (+)
9	Leaf deformation	0.182 (-)
10	Curling, cupping, puckering	0.146 (-)
11	Shoe stringing, mosaic	0.986 (+)
13	Leaf deformation, mosaic, puckering	0.994 (+)
14	Dwarfing, curling, mosaic	1.024 (+)
15	Mosaic, puckering	0.975 (+)
16	Yellowing, cupping	0.711 (+)
17	Mosaic, mottle, leaf deformation	0.728 (+)
18	Cupping, puckering	0.640 (+)
19	Dwarfing, mosaic, yellowing	0.559 (+)
20	Mosaic, cupping	0.761 (+)
21	Cupping, curling, mottle	0.620 (+)
22	Leaf deformation, yellowing	0.200 (-)
23	Yellowing, mosaic	0.189 (-)
24	Leaf deformation, puckering	0.511 (+)
25	Shoe stringing, cupping	0.662 (+)
26	Mosaic, cupping, curling	0.074 (-)
27	Leaf deformation	0.116 (-)
28	Mosaic, cupping	0.147 (-)
29	Cupping, curling, yellowing	0.154 (-)
30	Dwarfing, mosaic	0.493 (+)
31	Mottle, mosaic, leaf deformation	0.515 (+)
32	Shoe stringing, dwarfing	0.842 (+)
33	Mosaic, dwarfing, leaf deformation	0.963 (+)
34	Yellowing	0.171 (-)
35	Mosaic, leaf deformation	0.076 (-)
36	Puckering, mosaic, dwarfing	1.210 (+)
37	Shoe stringing, mosaic	0.999 (+)
38	Leaf deformation, mosaic, mottle	0.975 (+)
39	Leaf deformation, leaf curling, puckering	1.028 (+)
40	Yellowing, leaf deformation	0.260 (-)
41	Mosaic	0.243 (-)
42	Mottle, leaf deformation	0.141 (-)
43	Yellowing	0.172 (-)
44	Shoe stringing, mosaic	0.880 (+)
46	Puckering, leaf deformation, mosaic	0.713 (+)
47	Mosaic, curling	0.622 (+)
48	Yellowing, mosaic, leaf deformation	0.728 (+)
50	Leaf deformation	0.159 (-)
51	Mosaic	0.116 (-)
52	Leaf deformation, mosaic	0.103 (-)
53	Mottle, puckering	0.127 (-)
54	Mosaic, leaf deformation, puckering	0.771 (+)
55	Leaf deformation, yellowing	0.864 (+)
56	Yellowing	0.190 (-)
57	Leaf deformation, puckering	0.203 (-)
58	Mosaic, leaf deformation, dwarfing	0.668 (+)
59	Shoe stringing, mosaic	0.514 (+)
60	Leaf deformation, cupping	0.586 (+)
61	Mosaic, mottle	0.721 (+)
62	Leaf deformation, puckering, cupping	0.585 (+)

Table 2: Continue

Serial numbers	Symptoms	O.D. A_{405} nm
63	Cupping, dwarfing, mosaic	0.716 (+)
64	Yellowing, mottle	0.233 (-)
65	Leaf deformation, mottle	0.186 (-)
66	Mosaic, yellowing, cupping	0.689 (+)
67	Leaf deformation, yellowing	0.597 (+)
68	Cupping	0.211 (-)
69	Dwarfing, cupping	0.300 (-)
70	Leaf deformation	0.187 (-)
71	Mosaic, mottle, dwarfing	0.714 (+)
72	Shoe stringing, mosaic, cupping	0.866 (+)
73	Leaf deformation, mosaic	0.078 (-)
74	Yellowing, mosaic	0.154 (-)
75	Leaf deformation, mottle	0.491 (+)
76	Yellowing, mottle, mosaic	0.528 (+)
77	Leaf deformation, yellowing	0.246 (-)
78	Yellowing, mosaic	0.198 (-)
79	Cupping, puckering, mosaic	0.180 (-)
80	Mottle, leaf deformation	0.201 (-)
SOLAN		
1	Mosaic, mottle, dwarfing	0.399 (+)
2	Leaf deformation, yellowing	0.241 (-)
3	Puckering, yellowing	0.183 (-)
4	Mosaic	0.162 (-)
5	Yellowing, puckering, leaf deformation	0.099 (-)
6	Mosaic	0.724 (+)
7	Mottle, leaf deformation	0.522 (+)
8	Yellowing, dwarfing	0.717 (+)
9	Shoe stringing, mosaic	0.636 (+)
10	Puckering, leaf deformation, mosaic	0.143 (-)
11	Mosaic, cupping, leaf deformation	0.186 (-)
12	Mottle, cupping	0.515 (+)
13	Leaf deformation, dwarfing	0.489 (+)
15	Mosaic	0.116 (-)
16	Dwarfing, leaf deformation	0.128 (-)
17	Cupping, mosaic	0.311 (-)
18	Leaf deformation, curling	0.219 (-)
19	Mottle, cupping	0.260 (-)
20	Mosaic, mottle, leaf deformation	0.231 (-)
21	Dwarfing, mosaic	0.301 (-)
22	Curling, mosaic	0.194 (-)
23	Leaf deformation, mosaic, dwarfing	0.518 (+)
24	Mosaic, puckering	0.606 (+)
25	Dwarfing, cupping, mosaic	0.432 (+)
26	Mosaic, curling	0.617 (+)
27	Leaf deformation, yellowing	0.180 (-)
28	Cupping, yellowing	0.176 (-)
29	Leaf deformation, mosaic	0.243 (-)
30	Cupping, curling, mosaic	0.201 (-)
31	Mosaic, dwarfing	0.196 (-)
32	Mosaic, leaf deformation	0.040 (-)
33	Mottle, yellowing	0.117 (-)
34	Dwarfing, leaf deformation	0.181 (-)
35	Mosaic, cupping, shoe stringing	0.258 (-)
36	Mottle, cupping	0.221 (-)
37	Puckering, yellowing	0.477 (+)
38	Yellowing, leaf deformation	0.404 (+)
39	Mosaic, vein clearing	0.398 (+)
40	Leaf deformation, dwarfing	0.465 (+)

Table 2: Continue

Serial numbers	Symptoms	O.D. A_{405} nm
41	Mosaic, cupping	0.241 (-)
42	Leaf deformation, mosaic	0.183 (-)
43	Mottle, cupping	0.162 (-)
44	Leaf deformation, dwarfing	0.099 (-)
45	Mosaic	0.721 (+)
46	Dwarfing, leaf deformation	0.522 (+)
47	Shoe Stringing, mosaic, puckering	0.715 (+)
48	Curling, mosaic	0.636 (+)
49	Leaf deformation, yellowing	0.143 (-)
50	Yellowing, curling	0.186 (-)
51	Puckering, mosaic	0.515 (+)
52	Dwarfing, mosaic, leaf deformation	0.485 (+)
53	Leaf deformation, puckering	0.116 (-)
54	Yellowing	0.126 (-)
55	Mosaic, puckering	0.311 (-)
56	Curling, dwarfing, mosaic	0.219 (-)
57	Leaf deformation, mosaic	0.260 (-)
58	Vein clearing, curling	0.231 (-)
59	Mosaic, leaf deformation, dwarfing	0.301 (-)
60	Mottle, cupping	0.194 (-)
61	Puckering, yellowing	0.518 (+)
62	Yellowing, leaf deformation	0.606 (+)
63	Mosaic, vein clearing	0.432 (+)
64	Leaf deformation, dwarfing	0.617 (+)
65	Mosaic, cupping	0.180 (-)
66	Leaf deformation, mosaic	0.176 (-)
67	Mottle, cupping	0.243 (-)
68	Leaf deformation, dwarfing	0.201 (-)
69	Mosaic	0.196 (-)
70	Yellowing, leaf deformation	0.040 (-)
71	Dwarfing, mosaic	0.117 (-)
72	Shoe stringing, yellowing	0.181 (-)
73	Mosaic, cupping	0.258 (-)
74	Mottle, leaf deformation	0.221 (-)
75	Shoe stringing, mosaic, leaf deformation	0.477 (+)
76	Leaf deformation	0.404 (+)
77	Curling, cupping, puckering	0.398 (+)
78	Shoe stringing, mosaic	0.465 (+)
79	Leaf deformation, mosaic, puckering	0.556 (+)
80	Mosaic, vein clearing, curling	0.381 (+)
SIRMOUR		
1	Mottle, puckering	0.124 (-)
2	Mosaic, leaf deformation, puckering	0.177 (-)
3	Leaf deformation, yellowing	0.196 (-)
4	Yellowing	0.115 (-)
5	Leaf deformation, puckering	0.421 (+)
6	Mosaic, leaf deformation, dwarfing	0.409 (+)
7	Shoe stringing, mosaic	0.064 (-)
8	Leaf deformation, cupping	0.183 (-)
9	Mosaic, mottle	0.231 (-)
10	Leaf deformation, puckering, cupping	0.146 (-)
11	Cupping, dwarfing, mosaic	0.344 (+)
12	Yellowing, mottle	0.318 (+)
13	Leaf deformation, mottle	0.208 (-)
14	Mosaic, yellowing, cupping	0.193 (-)
15	Leaf deformation, yellowing	0.414 (+)
17	Cupping	0.407 (+)
18	Dwarfing, cupping	0.300 (-)

Table 2: Continue

Serial numbers	Symptoms	O.D. A_{405} nm
19	Leaf deformation	0.289 (-)
20	Mosaic, mottle, dwarfing	0.402 (+)
21	Shoe stringing, mosaic, cupping	0.390 (+)
22	Leaf deformation, mosaic	0.406 (+)
23	Shoe stringing, mosaic	0.413 (+)
24	Leaf deformation, dwarfing, cupping	0.395 (+)
25	Mosaic, curling, puckering	0.410 (+)
27	Shoe stringing, mosaic, dwarfing	0.401 (+)
28	Dwarfing, cupping, mosaic	0.375 (+)
29	Puckering, mosaic, curling	0.261 (-)
30	Leaf deformation, yellowing	0.285 (-)
31	Cupping, yellowing	0.113 (-)
32	Leaf deformation, mosaic	0.182 (-)
33	Cupping, curling, mosaic	0.180 (-)
34	Mosaic, dwarfing	0.210 (-)
35	Mosaic, leaf deformation	0.194 (-)
36	Mottle, yellowing	0.166 (-)
37	Dwarfing, leaf deformation	0.228 (-)
38	Mosaic, cupping, shoe stringing	0.301 (-)
39	Dwarfing, mottle, cupping	0.217 (-)
40	Puckering, yellowing	0.544 (+)
41	Yellowing, leaf deformation	0.512 (+)
42	Yellowing, mosaic, vein clearing	0.255 (-)
43	Leaf deformation, dwarfing	0.276 (-)
44	Mosaic, cupping	0.148 (-)
45	Leaf deformation, mosaic	0.089 (-)
46	Mottle, cupping	0.118 (-)
47	Leaf deformation, dwarfing	0.245 (-)
48	Mosaic	0.466 (+)
49	Dwarfing, leaf deformation	0.436 (+)
50	Shoe Stringing, mosaic, puckering	0.184 (-)
51	Curling, mosaic	0.183 (-)
52	Leaf deformation, yellowing	0.346 (+)
53	Yellowing, curling	0.407 (+)
54	Curling, mottle	0.211 (-)
55	Mosaic	0.193 (-)
56	Leaf deformation, mosaic	0.174 (-)
57	Vein clearing, mosaic, cupping	0.172 (-)
58	Mosaic, leaf deformation	0.146 (-)
59	Dwarfing, yellowing, mosaic	0.232 (-)
60	Cupping, mottle	0.228 (-)
61	Leaf deformation, vein clearing	0.106 (-)
62	Shoe stringing, mosaic	0.091 (-)
63	Mosaic, cupping	0.214 (-)
64	Puckering, mosaic, vein clearing	0.306 (-)
65	Mottle, phyllody	0.208 (-)
66	Curling, mosaic, leaf deformation	0.173 (-)
67	Vein clearing, cupping	0.181 (-)
68	Mosaic, leaf deformation, mottle	0.421 (+)
69	Puckering, mosaic	0.392 (+)
70	Vein clearing, dwarfing	0.105 (-)
71	Leaf deformation, cupping, mosaic	0.139 (-)
72	Yellowing, dwarfing	0.252 (-)
73	Shoe stringing, mosaic	0.341 (+)
74	Mosaic, puckering, leaf deformation	0.432 (+)
75	Mosaic, leaf deformation	0.150 (-)
Positive control		1.214 (+)
Negative control		0.180 (-)

O.D.: Optical density, A_{405}: Absorbance and nm: Nanometer

Similarly, O.D. values obtained for whiteflies confirmed their association with TYLCV as vectors and non-vectors for virus transmission in the surveyed fields. A maximum O.D. for *B. tabaci* of 0.725 was recorded from Charhatgarh in district Una followed by 0.501 from Sai in district Solan. However, *T. vaporariorum* tested negative for TYLCV with an O.D. value of 0.118 recorded from district Sirmour (Table 3).

Polymerase chain reaction assay for detecting whitefly species: Total DNA was successfully isolated from the collected whiteflies and was quantified as 1.89 for *B. tabaci* and 1.93 for *T. vaporariorum* at 260/280 nm. mtCOI gene primers were found capable enough to amplify the DNA fragments of both these whitefly species and were analyzed in 1% agarose gel. Bands of ~614 bp in *B. tabaci* and ~295 bp in *T. vaporariorum* resulted in characterizing the two whitefly species at a molecular level (Fig. 2).

Transmission efficiency of *Bemisia tabaci* and *Trialeurodes vaporariorum*: Tomato plants that tested positive for TYLCV were used to carry out transmission studies and a number of both whitefly species varying from 01-25 were allowed to feed on them with the same acquisition period of 04 hrs but a different inoculation access period of 04-24 hrs. *Bemisia tabaci* was found to be more efficient in transmitting TYLCV in comparison to *T. vaporariorum* as observed by the puckering and mosaic symptoms produced by the *B. tabaci* inoculated plants after 12-14 days of inoculation and O.D. values obtained after DAS-ELISA further confirmed the results. Maximum O.D. of 0.902 was recorded in the case of *B. tabaci* where 25 vectors transmitted TYLCV within 04 hrs of the acquisition access period and 24 hrs of the inoculation access period and a minimum of 0.431 where even one vector transmitted TYLCV within 04 hrs each of acquisition access period and inoculation access period whereas on the other hand in case of *T. vaporariorum* inoculated plant with 25 vectors with 4 hrs of acquisition access period and 24 hrs of inoculation access period failed to transmit TYLCV as observed from the O.D. values which is less than the negative control resulting in no transmission (Table 4).

Table 3: Serological assay of TYLCV in whiteflies using DAS-ELISA

Serial number	Locations	Whitefly (morphological trait)	O.D. A_{405} nm
1	Haroli, Una	*Bemisia tabaci*	0.518 (+)
2	Charhatgarh, Una	*Bemisia tabaci*	0.725 (+)
3	Sanjhot, Una	*Bemisia tabaci*	0.126 (-)
4	BanGarh, Una	*Bemisia tabaci*	0.484 (+)
5	Sai, Solan	*Bemisia tabaci*	0.501 (+)
6	Arki, Solan	*Bemisia tabaci*	0.224 (-)
7	Shamti, Solan	*Trialeurodes vaporariorum*	0.270 (-)
8	Giripul, Sirmour	*Trialeurodes vaporariorum*	0.065 (-)
9	Batol, Sirmour	*Trialeurodes vaporariorum*	0.118 (-)
10	Anji, Sirmour	*Trialeurodes vaporariorum*	0.093 (-)
Positive control			1.004 (+)
Negative control			0.169 (-)

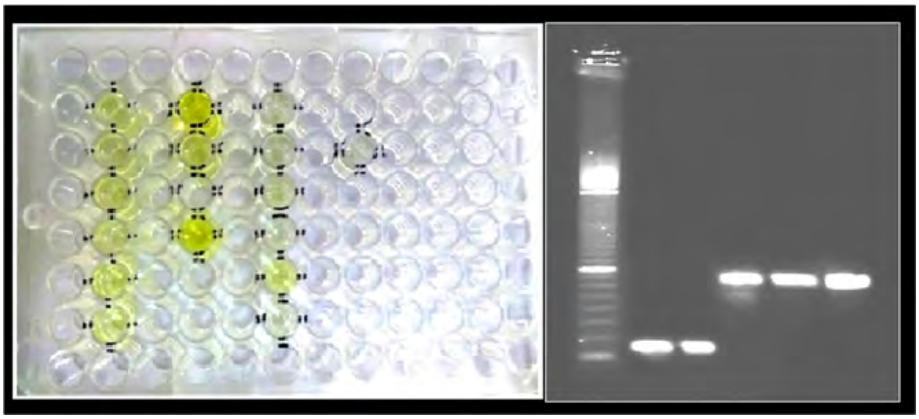

Fig. 2: Serological assays confirming the presence of TYLCV and molecular assay confirming *Bemisia tabaci* (~678 bp) and *Trialeurodes vaporariorum* (~2s10 bp)

Table 4: Serological assay for confirming transmission efficiency of whiteflies in a glasshouse

Serial numbers	Number of vectors		Acquisition feeding period	Inoculation access period	Number of plants showing symptoms (out of 10) O.D. A_{405} nm	
	B. tabaci	*T. vaporariorum*			*B. tabaci*	*T. vaporariorum*
1	01	01	04	04	03 (0.431)+	01 (0.121)-
2	05	05	04	08	03 (0.478)+	02 (0.135)-
3	10	10	04	12	05 (0.590)+	04 (0.212)-
4	15	15	04	16	07 (0.511)+	03 (0.187)-
5	20	20	04	20	07 (0.753)+	04 (0.194)-
6	25	25	04	24	09 (0.902)+	04 (0.205)-
Positive control					0.973 (+)	
Negative control					0.174 (-)	

DISCUSSION

Typical symptoms observed on tomato plants in the surveyed fields to record TYLCV were mosaic, mottle, yellowing, cupping, leaf deformation, curling, puckering, dwarfing and shoe stringing and similar types of symptoms were observed on tomato crops infected by TYLCV from many parts of the world[26-28], thereby, indicating that the observations were in line with these reports. The average incidence completely based on the visual symptoms recorded during the survey showed a minimum of 0.7 to a maximum of 95% of TYLCV. Visual symptoms have been the basis for collecting data on the incidence of TYLCV in many studies conducted around the globe such as in Pakistan at Mohmand Agency an incidence as high as 9.47% was recorded[29] and similarly, infection rates of TYLCV varied from 0.05-100% in greenhouse studies conducted in Turkey[30]. Similarly, the average whitefly population was calculated in the surveyed field on the marked symptomatic tomato plants and *B. tabaci* was encountered at 65.7% and *T. vaporariorum* at 62%. Whiteflies have always been found to be associated with TYLCV in tomato farms at varying ranges[29] and as in the present studies, the maximum occurrence of TYLCV in tomatoes was recorded in Charahatgarh along with the whitefly population too recorded the highest incidence at the same location. Similar findings have been reported from Pakistan where the maximum incidence of TYLCV (22.13%) in tomatoes and a high whitefly population was recorded from the same location Mohmand Agency[30].

Serological assays detected TYLCV not only in tomato plants but in the whitefly vector *Bemisia tabaci* as well that are present on infected plants in the surveyed fields and the DAS-ELISA technique has been successfully used for detecting TYLCV for its quick and reproducible nature from different parts of the world[31-35].

Molecular characterization done using the total DNA of both the whitefly vectors *B. tabaci* and *T. vaporariorum* in PCR assay contributes to finding the exact identity of the vector. Cassava-Colonizing *Bemisia tabaci* from eighteen African Countries (Burundi, Cameroon, Central African Republic (CAR), Democratic Republic of Congo (DRC), Madagascar, Nigeria, Rwanda, Tanzania, Benin, Ghana, Kenya, Liberia, Malawi, Mozambique, Sierra, Leone, Togo, Uganda and Zambia were collected and assayed using mtCOI gene primers also revealed the identity of *B. tabaci*[33]. The findings of the present studies go in line with many other researchers from the world[16,36-38] along with this another study conducted in Barcelona on *T. vaporariorum* where researchers used SCAR markers that resulted in the amplification of DNA with two separate bands of 2100 and 310 bp[39] PCR assays are therefore commonly used molecular strategies for determining the genetic trait of whitefly vectors.

Transmission studies conducted on tomato plants in controlled conditions regarding TYLCV movement by whitefly vectors explain the reason behind the spread of this virus in tomato fields and serological assays add affirmation to the study that helps in better understanding the virus-vector relationship. A similar type of work has been conducted by many workers keeping the idea of unravelling the virus-vector association that can help in the control of vector population and managing the spread of this threatening virus[29,30].

Serological evidence has confirmed the presence of TYLCV in symptomatic tomato plants along with whitefly vectors and molecular evidence helped in identifying the exact whitefly species associated with the virus. Besides, glasshouse studies revealed the transmission efficiency of these vectors. These strategies can be used to expand the host range for working out the transmission rate of these whitefly vectors in other crops as well.

CONCLUSION

The proliferation of *tomato yellow leaf curl virus* via *Bemisia tabaci* and *T. vaporariorum* was confirmed using serological assays in Una, Solan and Sirmour Districts of Himachal Pradesh. Molecular assay helped in characterizing the exact identity of whitefly species associated with TYLCV in the surveyed fields. Glasshouse studies made remarkable findings about both the whitefly species regarding their transmission efficiency and found *B. tabaci* as the major and only source of TYLCV transmission and *T. vaporariorum* was found to be non-viruliferous as none of the plants showed symptoms and tested negative in DAS-ELISA.

SIGNIFICANCE STATEMENT

Tomato is one of the important crops grown commercially throughout the world and the last decade has witnessed enormous expansion in area and production under this crop by small and commercial growers resulting from an ever-increasing demand. *Tomato yellow leaf curl virus* has been witnessed as a serious threat affecting the crop by declining its production. A comprehensive study based on extensive surveys, serological indexing of TYLCV in tomatoes as well as whitefly species associated including transmission studies to understand the virus-vector relationship that is expected to be of immense use for the farming community of Himachal Pradesh as *Bemisia tabaci* can be controlled well in time for suppressing the transmission whereas *Trialeurodes vaporariorum* which was found to be non-viruliferous is not a major concern.

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