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Tomato is one of the most economically important vegetable crops of India and its production in India is greatly hampered due to Tomato Leaf Curl Virus (ToLCV) which is one of the most damaging viral diseases of tomato worldwide. Detection of ToLCV from temperate region of Jammu and Kashmir was carried out using Polymerase Chain Reaction (PCR).The survey data revealed that the ToLCV was present in temperate zone of Jammu and Kashmir. The percentage of disease incidence ranged from 12.80% to 23.00% respectively. Maximum incidence (23.00%) of ToLCV was observed in Srinagar while minimum incidence (12.80%) was observed in Baramulla district. In Budgam district, the disease incidence of 15.20% was reported. Amplicons of size 700bp were obtained from the infected samples collected from different locations. The three amplified-PCR products (each representative of Srinagar, Baramulla and Budgam districts) were sequenced and the sequences were submitted to NCBI database and the accession numbers *viz.* KY-094937, KY-094936 and KY-094939 were obtained respectively. Phylogenetic analysis suggested that ToLCV Srinagar and Baramulla isolates clustered in Group 1 with Tomato leaf curl New Delhi virus isolate X1A segment DNA-A, Tomato leaf curl New Delhi virus isolate eggplant segment DNA-A and Tomato leaf curl New Delhi virus isolate parthenium segment DNA-A complete sequence whereas the ToLCV Budgam isolate clustered in Group III.

Key words: Tomato leaf curl virus, polymerase chain reaction, disease incidence, amplicon, phylogenetic analysis

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important and most widely grown vegetable crop of both tropics and sub tropics of the world, belonging to the family *Solanaceae* and ranks second in importance among vegetables. It is grown for its edible fruit which can be consumed either raw or cooked or in the form of various processed products like juice, ketchup, sauce, pickle, pastes, puree and powder. It is esteemed as an important source of vitamins A and C. In the world tomato is cultivated over an area of 28.52 lakh hectares with an annual production of 882.40 lakh tonnes (Anon., 1999). In India it occupies an area of about 5.40 lakh hectares with the production of 76.00 lakh tonnes (Anon. 2005). Though, the area under tomato cultivation is high, the productivity (15 t/ha) is low, this attributed to the potential loss in yield due to number of diseases. Tomato production in India is under constant threat of tomato leaf curl disease caused by Tomato Leaf Curl Virus (ToLCV) belonging to the family Geminiviridae and genus Begomovirus. The incidence of ToLCV has become a major limiting factor and challenge to the farmers due to the availability of the insect vectors throughout the year and the presence of perennial tomato host plants and weeds which enables the carryover of the virus to tomato crops. This disease is the major constraint in improving tomato production in India (Vasudeva and Samraj, 1948; Sastry and Singh, 1973; Saikia and Muniyappa, 1989; Harrison *et al*. 1991). Tomato production in Jammu and Kashmir has suffered considerable losses in yield and fruit quality due to infections of Tomato Leaf Curl Virus (ToLCV) (Sodhi *et al.* 2016). The diseased plants look pale and stunted due to shortening of internodal length with more lateral branches resulting in a bushy appearance (Vasudeva and Sam Raj, 1948). The whitefly *Bemisia tabaci* (Gennadius) (Homoptera:

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Aleyrodidae) has been found to be the vector of the virus (Vasudeva and Sam Raj, 1948; Saikia and Muniyappa, 1989). It was important that viruses occurring in a specific geographical area were identified and characterized prior to developing sustainable, environment friendly disease management programmes (Green and Kim, 1991). The present study was conducted in Jammu and Kashmir to detect ToLCV diseases using PCR assay. The PCR product was further sequenced and the comparison was made with reported ToLCV sequences of India and adjoining countries.

MATERIALS AND METHODS

Survey

A survey was under taken to know the incidence of Tomato Leaf Curl Virus disease (ToLCV) in temperate zones of Jammu and Kashmir. Temperate zone comprising of the districts of Srinagar, Baramulla and Budgam were surveyed for the presence of virus. All the plants in the selected plot area (10 m x 10 m) of the fields were counted and the number of plants showing leaf curl symptoms was recorded separately to calculate per cent disease incidence. The overall disease incidence was recorded based on visual symptoms. The percentage of disease incidence was calculated by counting the number of diseased plants exhibiting characteristic symptoms of the disease and by using following formula

$$\text{Per cent disease incidence} = \frac{\text{No. of infected plants}}{\text{Total No. of Plants observed}} \times 100$$

Sample collection and DNA isolation

Infected tomato leaf samples were placed in labeled polyethylene bags and transported to the laboratory for processing. The DNA from the infected leaf samples was later isolated. 100 mg of leaf tissue was crushed in liquid nitrogen and was incubated with 850 µl of CTAB (pH 8.0) at 65°C in serological water bath for 1hr with regular inversions. After that 24:1 solution of chloroform: isoamyl alcohol was added to separate out the chlorophyll and protein content after centrifugation at 10,000 g for 20 min. The supernatant was mixed with 650 µl of isopropyl alcohol to precipitate the DNA. After centrifugation, this precipitated DNA was washed with 70% and 100 per cent alcohol and after complete drying of ethanol; the DNA was dissolved in 100 µl of 1x TE solution and was stored at -20°C for further analysis.

PCR detection

For the detection of Tomato Leaf Curl Virus, the PCR was performed in a 25 µl reaction mix where 2 µl of the DNA taken as template was amplified .The reaction mix finally consisting of 200 µM each dNTPs, primers (20 pmol),1.5 mM $MgCl_2$ and 10 X PCR buffer. The PCR cycles set were, initial one cycle of denaturation at 94°C of 5 min followed by 35 cycles of denaturation at 94°C (1 min), annealing at 50°C (1 min) and extension at 72 C for 1min; the final extension was given at 72°C for 10 min.

Gel Electrophoresis

The amplified PCR products were analyzed on one per cent agarose gel containing ethidium bromide (10 mg ml^{-1}).The agarose gel (1%) was prepared in 1X TAE solution and the samples were electrophoresed by supplying voltage @ 5V cm^{-1}. The 1 kb ladder was used as marker to identify the size of the amplified product.

Sequencing of Amplified PCR Fragments

Amplified PCR fragements obtained from the infected ToLCV samples from Srinagar, Baramulla and Budgam were sequenced (Chromus Biotech India Pvt. Ltd., Bangalore). The sequences were submitted to NCBI database (www.ncbi.nlm.nih.gov).

Phylogenetic analysis

Alignments of sequences of ToLCV Srinagar, Baramulla and Budgam isolates with other sequences of ToLCV available in the NCBI database was made using ClustalW with default parameters. Unrooted phylogenetic trees were constructed with MEGA 6 (http://www.megasoftware.net/) using the Neighbor-Joining (NJ) method with Poisson correction, pairwise deletion and 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Disease incidence of Tomato Leaf Curl Virus from temperate zone of Jammu and Kashmir

The survey data revealed that the virus was present

in all the locations surveyed. The percentage of disease incidence ranged from 12.80 to 23.00 respectively (Table1). Maximum incidence of ToLCV (23.00%) was recorded in Srinagar while minimum incidence (12.80%) was observed in in Baramulla district. In Budgam district, the percentage of disease incidence was 15.20% (Table 1 ; Fig. 1). The results revealed that the per cent disease incidence varied from location to location. The variation in disease incidence over locations might be due to the source of virus inoculum, presence of weed hosts, local variation in temperature and relative humidity that may have direct influence on vector population and its migration, the effect of climate on biology and distribution of vector. However, the disease (ToLCV) and the vector, *Bemisia tabaci* were found in almost all the tomato fields surveyed. The higher incidence during summer months could be attributed to higher whitefly population and high temperature. Seetharama Reddy (1978) also observed 6-38 per cent ToLCV incidence during winter months and 25-86 per cent during summer months. Similar observations were made by Saikia and Muniyappa (1989) and Raghavendra (2002) who recorded 24 -100 per cent ToLCV incidence. Singh and Reddy (1993) reported 60-100 per cent ToLCV incidence from tomato growing areas of Karnataka.

Table 1: Percent disease incidence of Tomato leaf curl disease from different locations of Kashmir region

Location	Percent Disease Incidence (%)
Srinagar	23.00
Baramulla	12.80
Budgam	15.20

Table 2: Sequences of ToLCV isolates from Srinagar, Baramulla and Budgam

Name of the Virus	Location	Nucleotide Sequence	Accession No.
ToLCV Srinagar isolate	Srinagar	CCAGCAGATATCATCATTTCAACTCCCGCATCGAAAGTACGCCGACGTCTC AACTTCGACAGCCCCTATGGAGCTCGTGCAGTTGTCCTCATTGCCCGCGTC ACAAAAGCAAAGGCCTGGGCCAACAGGCCGATGAACAGAAAACCCAGAAT GTACAGAATGTATAGAAGTCCCGACGTGCCAAGGGGTTGTGAAGGCCCTT GTAAGGTGCAATCCTTTGAATCCAGGCACGATGTATCTGATATTGGTAAAGT CATGTGTGTTAGTGATGTTACCCGAGGAACCGGACTCACACATCGGTAGGG AAGCGATTCTGTGTGAAATCTGTCTACGTCCTGGGAAAGATATGGATGGAT GAAAACATCAAGACGAAAAACCATACTAACAGTGTTATGTTTTTTTTAGTTCG TGACCGTCGCCTACAGGAACCCCGCAAGATTTCGGGGAAGTGTTTAATATG TTTGACAATGAACCGAGCACAGCAACGGTGAAGAACATGCATCGTGATCGT TATCAAGTCTTACGGAAGTGGCATGCAACTGTGACCGGAGGAACATACGCA TCTAAGGAGCAAGCAT	KY-094937
ToLCV Baramulla isolate	Baramulla	AACTATGGCGAAGCGACCAGCAGATATCATCATTTCAACTCCCGCATCGAA AGTACGCCACGTCTCAACTTCGACAGCCCCTATGGAGCTCGTGCAGTTGTC CCCATTGCCCGCGTCACAAAAGCAAAGGCCTGGGCCAACAGGCCGATGAA CAGAAAACCCAGAATGTACAGAATGTATAGAAGTCCCGACGTGCCAAGGG GTTGTGAAGGCCCTTGTAAGGTGCAATCCTTTGAATCCAGGCACGATGTAT CTCATATTGGTAAAGTCATGTGTGTTAGTGATGTTACCCGAGGAACGGACT CACACATCGCGTAGGGAAGCGATTCTGTGTGAAATCTGTCTACGTCCTGGG AAAGATATGGATGGATGAAAACATCAAGACGAAAAACCATACTAACAGTGTT ATGTTTTTTTTAGTTCGTGACCGTCGTCCTACAGGAACCCCGCAAGATTTCG GGGAAGTGTTTAATATGTTTGACAATGAACCGAGCACAGCAACGGTGAAGA ACATGCATCGTGATCGTTATCAAGTCTACGGAAGTGGCATGCAACTGTGAC CGGAGGAACATACGCA	KY-094936
ToLCV Budgam isolate	Budgam	TACTATGGCGAAGCGACCAGCAGATATCATCATTTCAACTCCCGCATCGAA AGTACGCCGACGTCTCAACTTCGAGGGGTTGTGAAGGCCCTTGTAAGGTG CAATCCTTTGAATCCAGGCACGATGTATCTCATATTGGTAAAGTCATGTGTG TTAGTGATGTTACCCGGGAACCGGACTCACACATCGCGTAGGGAGCGATT CTGTGTGAAATCTGTCTACGTCCTGGGAAAGATATGGATGGATGAAAACAT CAAGACGAAAAACCATACTAACAGGTTATGTTTTTTTTAGTTCGTGACCGTC GTCCTACAGGAACCCCGCAAGATTTCGGGGAAGTGTTTAAATGTTTGACAA TGAACCGAGCACAGCAACGGTGAAGAACATGCATCGTGATCGTTATCAAGT CTTACGGAAGTGGCATGCAACTGTGACCGGAGGAACATACGCATCTAAGG AGCAAGCATTAGTTAGGA AG	KY-094939

Molecular identification of Tomato Leaf Curl Virus from temperate zone of Jammu and Kashmir

Tomato leaf curl viral DNA was amplified in PCR using ToLCV specific primers, AV1 F: ATGGCGAAGCGACCAG and AV2 R: TTAATTTGTGACCGAATCAT. The virus was detected through PCR from DNA of infected samples

which showed amplification of size 700bp after gel electrophoresis which was further re-evaluated (Fig.2). Polymerase Chain Reaction (PCR) method amplifies a specific DNA sequence that is present between two regions of known nucleotide sequence (Innies *et al.* 1990) and is widely used. PCR technique over comes problems associated in serological detection methods and has been utilized successfully to detect geminiviruses (Deng *et al.* 1994; Rojas *et al.* 1993 ; Mehta *et al.* 1994).

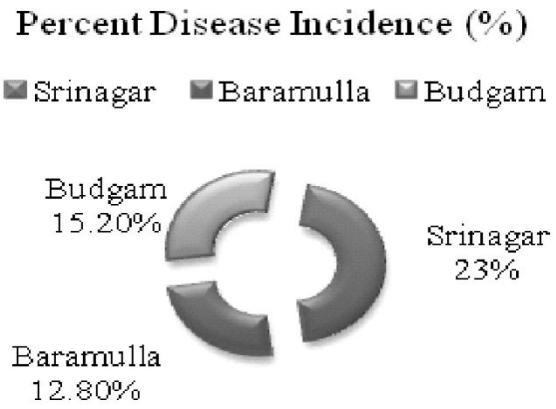

Fig. 1 : Percent disease incidence of Tomato leaf curl disease from different locations of Kashmir region

The three amplified-PCR fragments each representative of Srinagar, Baramulla and Budgam districts were sequenced and the sequences were submitted to NCBI database (www.ncbi.nlm.nih.gov) and the accession numbers *viz.* KY-094937, KY-094936 and KY-094939 were obtained respectively (Table 2). Dendrogram was constructed based on the nucleotide sequence similarity data for ToLCV available on NCBI database using ToLCV isolates of Srinagar, Baramulla and Budgam as reference using the Neighbor-Joining (NJ) method with Poisson correction, pairwise deletion and 1000 bootstrap replicates using MEGA 6 software (http://www.megasoftware.net/). The dendrogram produced three major clusters (Fig. 3). Group I cluster comprises of Tomato leaf curl New Delhi virus isolate X1A segment DNA-A, Tomato leaf curl New Delhi virus isolate eggplant segment DNA-A, Tomato leaf curl New Delhi virus isolate parthenium segment DNA-A complete sequence, Tomato Leaf Curl Virus Srinagar isolate and Tomato Leaf Curl Virus Baramulla isolate. Group II cluster comprises of Tomato leaf curl New Delhi virus segment A, clone 14AA3-11RS99, Tomato Leaf Curl Virus isolate Himachal coat protein gene, complete Cds and Tomato leaf curl New Delhi virus complete segment DNA-A, isolate Lahore. Group III cluster include the remaining Tomato Leaf Curl Virus Budgam isolate. Cluster analysis also revealed that isolates under group I are found to have a close relationship among themselves whereas Group III (Tomato Leaf Curl Virus Budgam isolate) was found different from rest of the ToLCV isolates under study. This gives an indication that the ToLCV Srinagar isolate and ToLCV Baramulla isolate has similarity with other ToLCV isolates already reported. Irrespective of the host and geographical origin the isolates are dispersed in the whole country indicating a single gene pool.

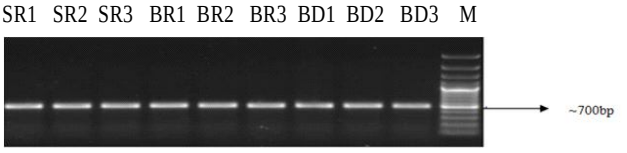

Fig. 2 : PCR Amplification (700bp) of viral coat protein with specific primers from infected samples collected from Srinagar (SR1-SR3), Baramulla (BR1-BR3) and Budgam (BD1-BD3) regions; M: 1Kb molecular marker

Based on the above data, it can be concluded that ToLCV infection is widely spread in all the locations surveyed crafting a definitive need to control and manage the virus. Various factors such as climatic conditions and cultural practices play a vital role in the level of infection. Level of infection in all fields falling under the same agro-climatic zone was found to be similar. Thus the results suggested that the PCR method enabled the detection of the ToLCV infecting tomato.

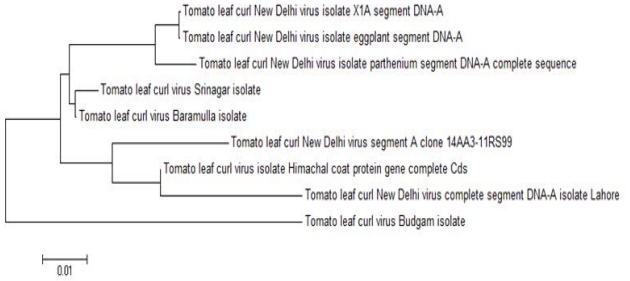

Fig. 3 : Dendrogram showing nucleotide similarity of Tomato leaf curl virus isolates of temperate zone of J&K with other isolates worldwide

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