Molecular characterization of tospoviruses associated with ringspot disease in bell pepper from different districts of Himachal Pradesh

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Abstract Bell pepper (*Capsicum annuum* L.), an important cash crop for the farmers of Himachal Pradesh was found to be affected with tospovirus like disease. An extensive survey was conducted in the bell pepper grown areas in the five districts of Himachal Pradesh to identify and characterize the causative agent. Hence, 60 symptomatic bell pepper plants exhibiting characteristics symptoms were collected from Solan, Sirmaur, Hamirpur, Kangra and Bilaspur districts. Out of 60 samples, 53 samples were found to be positive by DAS-ELISA with tospovirus group specific antiserum. To confirm the presence of tospovirus, DAC-ELISA was performed using GBNV/CaCV polyclonal antiserum and DAS-ELISA with two monoclonal antibodies i.e. TSWV, GRSV. All the 53 samples were found negative for TSWV and GRSV and positive for GBNV/CaCV. Further, eleven infected isolates from both poly-house and open field conditions were selected for characterization at molecular level. RT-PCR was performed with N gene specific primers for TSWV, GBNV and CaCV. The eleven samples selected for molecular identification were further found to be negative for TSWV and positive for CaCV using RT-PCR. One of the samples from district Sirmaur was found to be positive

for mixed infection of GBNV and CaCV. N gene phylogenetic analysis of CaCV/GBNV provided important information about the movement and evolution of tospoviruses in Himachal Pradesh.

Keywords *Capsicum chlorosis virus* · *Groundnut bud necrosis virus* · *Tomato spotted wilt spot virus* · Enzyme linked immunosorbent assay · Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Introduction

Capsicum (*Capsicum annuum* L. var. *grossum* Sendt), is cultivated in most parts of the world which is commonly known as bell pepper and sweet pepper. Cultivation of chilies and bell pepper in India spans around 8000 Hectares of land with an estimated production of 68,000 tons [6]. In Himachal Pradesh, it is cultivated in various agro climatic zones as off season crop (May–October) which covers an area of 2503 Hectares with a production of 33,923 tons including hot pepper [3]. Worldwide, virus infection leads to great economic loss to its cultivators. *Tomato spotted wilt virus* (TSWV) alone has been reported to cause \$ 1 billion loss annually [1]. A total of 68 viruses have been reported to infect pepper from various part of the world in terms of frequency of distribution, damage and host range. In India, 19 different viruses have been reported to cause natural infection of bell pepper [14]. The tospoviruses belong to the family *Bunyaviridae* which is of great economic concern for vegetable production globally [13]. Genome of tospoviruses is tripartite in nature having three segments named S (3 kb), M (4.8 kb) and L (9 kb) [12]. More than 20 different tospoviruses have been reported worldwide, out of which five tospoviruses -*Peanut bud*

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necrosis virus (PBNV) [16], *Watermelon bud necrosis virus* (WBNV) [9], *Capsicum chlorosis virus* (CaCV) [10], *Iris yellow spot virus* (IYSV) [5, 15] and *Peanut yellow spot virus* (PYSV) [17] have been reported so far from India. Symptoms of tospoviruses found in India are characterized by stunted growth of plants and yellow concentric rings on the leaves. Key characteristics of tospoviral infection i.e. concentric ring pattern have also been found in most of the capsicum growing areas of Himachal Pradesh. Keeping in mind the importance of tospoviruses, a survey was conducted to find out the kinds (TSWV, GRSV, GBNV and CaCV) of tospoviruses infecting capsicum in Himachal Pradesh. To study the relatedness of tospoviruses, phylogenetic analysis was also executed in the present research using N gene/protein sequences obtained from various isolates throughout the world.

During the year 2012–2013, five districts of Himachal Pradesh were monitored for the incidence of tospoviruses on selected bell pepper plantations and disease incidence was recorded [4]. Infected leaf samples were collected from polyhouses and open field grown capsicum. Collected leaf samples were transported in liquid nitrogen containers from the infected fields to the laboratory and were stored at -80°C . A total of 30 villages have been surveyed in five districts in which 10 villages from district Sirmaur, 7 from Bilaspur, 5 villages from Solan, 5 from Hamirpur and 3 villages from Kangra districts were included. From Kangra district, bell pepper samples were collected from the capsicum grown in polyhouse only and from Bilaspur district, samples were collected from open fields only; besides it, from the rest of the districts, samples were collected both from open fields and polyhouses. Total of 60 bell pepper plants with characteristic symptoms like chlorotic concentric ringspot, necrotic concentric ringspot, and distortion of leaves and stunted growth of plants were collected both from protected environment as well as open field conditions spanning in five districts of Himachal Pradesh (Fig. 1).

The virus incidence in collected samples was first analyzed serologically using tospovirus group specific antibody (BioREBA, Switzerland), monoclonal TSWV and GRSV specific antibody (Agdia) and polyclonal antibody against GBNV/CaCV (obtained from Department of Plant Pathology, IARI New Delhi) following supplier's protocols. Absorbance was taken at 405 nm using ELISA reader (BIORAD, USA) and samples higher than fivefold value than the negative control (a healthy capsicum plant) were considered positive.

For DAS-ELISA, primary and alkaline phosphatase labeled antibodies (raised against the specific virus) used were provided with BIOREBA kit. For DAC-ELISA primary antibody (rabbit anti CaCV: obtained from Department of Plant Pathology, IARI) and alkaline phosphatases labeled anti rabbit secondary antibody (SIGMA) was used. Substrate

for alkaline phosphatase (p-nitrophenyl phosphate) was procured from sigma. In addition negative (healthy capsicum) and positive controls provided in the kit (BIOREBA) were used with all the tests. Young leaves of selected ELISA positive bell pepper plants were taken and total RNA was extracted from the infected leaves using one step RNA reagent (Biobasic, Inc) with little modification in manufacturer's instructions. Total RNA was used as a template to synthesize cDNA using virus specific reverse primer and superscript II reverse transcriptase (Invitrogen) in the reverse transcription-polymerase chain reaction (RT-PCR), as per manufacturer's instructions. First strand cDNA was used to generate the second strand and for the amplification of dsDNA in a PCR reaction for 1 cycle of denaturation at 95°C for 2 min and 35 cycles of PCR conditions at 95°C denaturation (30 s), 54°C annealing (30 s) and 72°C extension (1 min) and one cycle of final extension at 72°C for 10 min. PCR reaction was carried out in automated thermo cycler (Applied Biosystems) with 25 μl of total reaction mixture containing 2 μl of cDNA product, 1 μl of upstream (forward) primer (20 pmol/ μl), 1 μl of downstream (reverse) primer (20 pmol/ μl ; for all the tospoviruses selected as shown in Table S1), 2.5 μl of $10 \times$ Taq DNA polymerase assay buffer (Bangalore Genei) with 15 mM MgCl_2 , 2 μl of 1.5 mM dNTP mix and 0.5 μl of Taq DNA polymerase (Bangalore Genei; 3 units/ μl). Amplified products were analyzed using 1 % agarose gel in Tris–Acetic acid-EDTA (TAE) buffer with DNA ladder (1 kb plus Fermentas) as marker. The PCR products were purified using QIAquick gel extraction kit (Qiagen). Two samples (except Kangra from where all the three samples were selected) from each district representing both polyhouse and open field conditions were selected and sequenced (Xcelris genomics). All the three samples of Kangra district and two of Bilaspur district grown in polyhouse and open field respectively were also selected. The sequences were aligned with corresponding sequences of other established tospovirus sequences from the database using BLAST from the website <http://www.ncbi.nlm.nih.gov/blast> [2].

During the survey, maximum characteristic symptoms of tospovirus infection were observed in bell pepper grown in protected environment (Polyhouse).

Out of five, characteristic symptoms (concentric ring pattern; Fig. 1) were recorded at three districts viz. Sirmaur, Hamirpur and Kangra along with diffused ring symptoms on young leaves of some plants grown in polyhouse of Kangra district (Fig. 1). The disease incidence based on viral symptoms which varied from 0–80 % in different villages surveyed in five districts (Sirmaur-80 %, Kangra-80 %, Bilaspur-80 %, Hamirpur-50 % and Solan-25 %) of Himachal Pradesh. Absence or very less disease incidence observed at few locations could be due to chemical control methods used against thrips or

Fig. 1 Bell pepper leaves showing characteristic symptoms of tospoviruses from five districts of Himachal Pradesh. **a** Chlorotic concentric ringspot symptom observed from sample collected from District Sirmaur; **b** Diffused chlorotic ringspot symptom observed from sample collected from District Kangra; **c** Chlorotic ringspot symptom observed from sample collected from District Bilaspur; **d** Diffused chlorotic ringspot symptom observed from sample collected from District Hamirpur

phytosanitary practices used by some of the farmers. Other contributing causes could be absence of thrips in a particular area or non-transmission of viruses. Serological assessment was confirmed by using both Direct Antigen Coating-Enzyme linked immunosorbent assay (DAC-ELISA) and Double Antibody Sandwich-Enzyme linked immunosorbent assay (DAS-ELISA). Out of 60, 7 leaf samples (from Solan district) were found to be negative for all tospoviral serums (Tospovirus group specific, TSWV, GRSV and GBNV/CaCV). Necrotic lesions observed in these samples at the time of collection was probably because of other pathogens, physiological disorders, environmental factors or some unidentified viruses as also reported earlier [8]. Furthermore, it has also been reported earlier that the combined infection of several viruses can change the symptoms expression [7]. All the 60 samples from different districts were found to be negative for TSWV and GRSV, hence ruling out the possibility of these two viruses in bell pepper samples of Himachal Pradesh. Out of 60, 53 samples were found positive for CaCV/GBNV by serological identification. Symptomatic bell pepper samples C1, T (Sirmaur), H1, E2 (Hamirpur), R1, Chb (Solan), J1, G5 (Bilaspur), I1, I2 and I4 (Kangra) which were consistently found positive with serological tests for CaCV/GBNV were used for molecular identification. RT-PCR was performed in three stages, first with TSWV specific primers, which was earlier found to be negative on the basis of serological identification. Secondly, RT-PCR was performed with GBNV specific

primers for the confirmation of the presence of GBNV in selected bell pepper isolates. No amplification was observed with first stage RT-PCR (not shown) with TSWV CP specific primers. But an amplification product was obtained in C1 isolate from district Sirmaur in second stage of RT-PCR with GBNV specific primers. It was observed that the rest of the 10 isolates used for the PCR reaction in second stage RT-PCR were also found to be negative for the presence of GBNV (not shown). Therefore, amplified product from C1 isolate was purified and sequenced. Sequence obtained (465 bp) from PCR amplified product of sample C1 was used for BLAST analysis and was found to be 95 % identical at nucleotide/amino acid level with GBNV-[Vum2-Knp] (JQ 406583) isolate reported from *Vigna* sp, India. In third stage RT-PCR with CaCV specific primers, amplification of ~800 bp was observed in all 11 isolates from different districts of Himachal Pradesh (Fig.S1; Table S2). Except for E2 isolate from district Hamirpur, amplified PCR products from rest 10 isolates were sequenced. Sequences obtained were used in BLAST analysis and showed identity ranging 98–99 % at nucleotide/amino acid level with CaCV isolates already reported from different geographical locations of the world (Table S2). The N gene/protein sequences identified in the study have been submitted at European Nucleotide Archive (ENA) and accession numbers have been obtained (Table S2).

To gain insight of the evolutionary pattern, a phylogenetic tree was constructed with isolates of CaCV and

Fig. 2 Phylogenetic relationship of 11 N gene/protein sequences of Himachali tospovirus isolates with previously reported isolates from India and other parts of the world

GBNV associated with bell pepper reported in the present study with those previously reported from other geographical regions of the world. The N gene/protein sequences reported from other parts of the world includes CaCV, GBNV (PBNV), TSWV and INSV infecting different crops causing various symptoms on their respective hosts. The tree was constructed by Neighbor-Joining (J) method using MEGA software version 6. Eleven (One GBNV and 10 CaCV) isolates from present study and 32 isolates reported earlier worldwide were grouped into four main clusters (Fig. 2). Himachal's CaCV isolates were grouped into two main clusters (Fig. 2). First cluster represents 10 CaCV isolates from this study and 22 CaCV isolates from the rest of the world. Bigger cluster have been divided into many clades. All the 10 isolates of CaCV confirmed from this study have been presented together in the first cluster and forming three

clades. Clade I represented two isolates G5 (537 bp) and J (764 bp) from Bilaspur, two isolates I1 (713 bp) and I4 (734 bp) from Kangra and one isolate T (734 bp) from district Sirmaur. Clade II was made up of two isolates i.e. one C1 (791 bp) from district Sirmaur and second CaCV from its natural host *Amaranthus* which was recently reported from India [18]. Clade III was produced from isolates H1 (747 bp from Hamirpur), I2 (767 bp from Kangra) and Chb (686 bp from Solan) from this study and two Indian-CaCV isolates (EU216023, FJ011449) were already reported. However, R1 isolate from district Solan (present study) did not make cluster with either CaCV isolate or with any other tospoviruses under study (Fig. 2). GBNV C1 (Accession no-LN875777) of this study clustered together with four Indian GBNV isolates (KF723559, JX524556, HM770022 and FJ355952) in third cluster. Fourth cluster was made up from TSWV

and INSV isolates of various crops from throughout the world.

It has been observed that mixed infection is not reported very frequently for tospoviruses, but during this finding one mixed infection (GBNV and CaCV) has been reported from C1 sample of district Sirmaur. In contrast, during earlier survey in northern states of India CaCV and PBNV were reported independently but not in mixed infections [11]. Sequence data obtained was used further for multiple alignment. As shown in Table S3, the values above diagonal represented percent homology of nucleotide sequences of 10 isolates. Maximum identity among nucleotides was found to be 99.86 % which was between isolates J1 isolate from Bilaspur district and T isolate from Sirmaur district and similar to this minimum homology was found to be 97.01 % between G5 isolate from Bilaspur district and I2 isolate from district Kangra (Table S3). Similar matrix, in phylogenetic tree also depicts that J1 isolate and T1 isolate showing the maximum identity (99.86 %) and fall in the same clade of cluster I. Multiple alignment of amino acid sequences of N protein/gene of 10 isolates of CaCV of present study were represented below diagonal (Table S3). Maximum identity (100 %) was found in many combinations like J1 from Bilaspur and Chb from Solan, R1 from Solan and H1 from Hamirpur, H1 from Hamirpur and Chb from Solan etc. and minimum (94.97 %) was found between C1 from Sirmaur and G5 from Bilaspur isolates (Table S3). Hence, based on various parameters i.e. serological and molecular methods used for identification, the virus causing disease of bell pepper under natural and controlled conditions in Himachal Pradesh was identified as *Capsicum chlorosis virus* (CaCV). Although out of 11 samples tested one was found to have mixed infection of both CaCV and GBNV.

Mixed infection of GBNV and CaCV has been reported from Badripur village of district Sirmaur. To the best of our knowledge, this is the first report of natural mixed infection in bell pepper from India. A comparative account of serological and molecular characterization of GBNV and CaCV reported in bell pepper isolates of different districts of Himachal Pradesh has been reported. This study confirms the presence of CaCV as prevalent tospovirus from Himachal Pradesh and it has also been reported that tospoviruses from Himachal have no or very less variations among themselves. Himachal CaCV isolates were closely related to CaCV isolates which were already reported from India.

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