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Detection of *Capsicum chlorosis virus* (CaCV), An Emerging Virus Infecting Chilli in Tamil Nadu, India

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Abstract

Chilli plants in the farmers' fields in Tamil Nadu showing symptoms of concentric chlorotic and necrotic rings on the leaves were found infected with a *Tospovirus* through RT- PCR by using *Tospovirus* degenerate primer (gL3637 /F and gL4435C). From the BLAST result the virus associated with the disease is identified as *Capsicum chlorosis virus* (CaCV). Infected samples collected from Coimbatore were amplified using newly designed primer pair (GKCaCVCPF1/R1) corresponding to the nucleocapsid region of the CaCV for further confirmation. In the sequence analysis, complete nucleocapsid protein region shared a nucleotide and amino acid identity of 99.1% and 100% with the PB4 and PB1 isolates of CaCV from India respectively. In the phylogenetic analysis, this isolate grouped with the CaCV isolates from Aurangabad and Tamil Nadu, India than with isolates from other countries. About fifteen hosts from different families were inoculated through sap, of which nine hosts produce local lesion and four showed systemic symptoms. The positive reaction of extract from these plants in DAS- ELISA (double antibody sandwich ELISA) against the nucleocapsid protein of CaCV (DSMZ, Germany), confirmed the presence of CaCV in infected chilli plants in Tamil Nadu.

Keywords

Chilli; CaCV, RT-PCR, DAS-ELISA; Host Range

Introduction

Chilli pepper (*Capsicum annuum* L.) is one of the most important spice crops in India. It is cultivated worldwide, especially in temperate regions of Central and South America and European countries, tropical and subtropical regions of Asian continent mainly in India and China. Though chilli is susceptible to several viral diseases, the necrosis disease caused by *Tospovirus* is very devastating. In India, *Tospovirus* have emerged as a serious threat to vegetable crops during the last 10 to 15 years [1]. Capsicum chlorosis virus (CaCV), a member of *Tospovirus* genus was reported for the first time in tomato in northern India during 2007 post-rainy season [2] and subsequently in chilli peppers in southern India, Karnataka [3]. In a survey conducted in Coimbatore district of Tamil Nadu, during 2014-15, concentric chlorotic and necrotic rings on the leaves were observed. The infected plants had a retarded growth when compared to the healthy ones. Results on host range study and identification and characterization of the virus associated with the chlorosis and necrotic spots on chilli crop in Tamil Nadu, are presented in this communication.

Materials and Methods

Survey and collection of the plant sample

Surveys were conducted during 2014-15 during Rabi season in different chilli growing areas of Coimbatore to assess and document the virus associated with the necrosis disease of chilli. During the survey, chilli plants showing concentric chlorotic and necrotic rings on the leaves were observed. The infected sample with the healthy sample were collected and brought to the laboratory in the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for molecular analysis of the associated virus and host range study. The samples were stored at -80°C until further studies [4].

Total RNA extraction and conversion of cDNA

The total RNA was isolated from the virus infected chilli samples as well as the healthy leaf samples by using Total Plant RNA extraction Kit (Sigma Aldrich, USA) according to the manufacturer's instruction. First strand cDNA synthesis was carried out using cDNA synthesis kit *(Revert Aid First Strand cDNA synthesis kit, Thermo Scientific, USA*) as per manufacturer's instruction. The reaction was performed at 42°C for 60 min followed by incubation at 70°C for 5 min. RT- PCR has been carried out with the Tospovirus universal primer pair (gl3617/F– CCTTTAACAGTDGAAACAT, gl4435c/R – CATDGCRCAAGARTG RTARACAGA) corresponding to the L segment of Tospovirus. PCR was carried out with the necrotic and chlorotic samples expressing both chlorotic and necrotic symptoms with the master mix (Smart Prime, India) in 50 µl reaction volume containing master mix- 25 µl; gl3617/F – 5 µl; gl4435c/R – 5 µl; distilled water – 10 µl; cDNA – 5 µl. The PCR products were sequenced and the sequence results were analysed in the BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A new primer pair (GKCaCVCPF1: AACCAATAGTTTGCCTCCG; GK CaCV CPR1: AGAGCAATCGAGGCACTA) was designed based on the nucleocapsid gene sequence of CaCV (Genbank accession KC953852) available in NCBI database in order to amplify the entire nucleocapsid protein gene. PCR reaction was standardized with the following conditions: Initial denaturation of 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 10 min. The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced [5-9].

Sequence comparison and phylogenetic analysis

The sequence of the virus isolate under this study was compared with the sequences of selected *Tospovirus* species obtained from the NCBI gene bank database. Multiple sequence alignment was done using Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 6.0 software (www.megasoftware.net) generated using Neighbour joining tree method, bootstrapped with 1000 replicates

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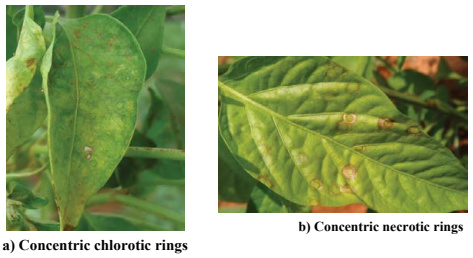

Figure 1: Symptoms of tospoviruses on chilli in Tamil Nadu.

Percentage identity was generated using ClustalW algorithm in Bio edit version 7.0.9.0.

Biological host range

The CaCV infected samples from chilli were used to study the biological host range. Fifteen plant species belonging to eight different families (Table 1) were mechanically inoculated with CaCV. The inoculated plants were monitored for the expression of symptoms up to 25–30 days post-inoculation (dpi) in an insect proof greenhouse. Local and systemic infections were confirmed by observing symptoms on inoculated and newly emerged leaves. The infected leaves were tested for the presence of virus by DAS-ELISA using PAbs specific for the CaCV Nc protein (DSMZ, Germany) [9]

Double antibody sandwich- enzyme linked immunosorbant assay DAS-ELISA

The virus infected chilli field samples showing virus symptoms and mechanically inoculated plant samples from different host were subjected to Double Antibody Sandwich- Enzyme Linked Immuno Sorbant Assay (DAS- ELISA) at 1: 500 dilutions of both the antibody and the anti-virus conjugate using a kit obtained from the DSMZ, Germany as per the procedure described by Clark and Adams [10,11]. The test sample values at least two or three times higher than the respective healthy controls were considered as positive.

Results and Discussion

During the survey in different chilli growing areas of Coimbatore, symptoms such as necrosis and chlorosis on the leaves observed in three different locations in Titheepalayam village (Figure 1) were recorded with disease incidence ranging from 10-40%.

Detection and characterization of the virus

RNA extracted from the symptomatic (chlorotic and necrotic) and non-symptomatic leaves of chilli were subjected to preliminary screening for the presence of *Tospovirus* using gl3617/F and gl4435c/R – primer pairs through RT-PCR assay. Symptomatic leaves showed an amplification of ~830bp; no amplicons were obtained from non-symptomatic leaves which confirm the presence of *Tospovirus* associated with the disease of chilli. Amplified products were sequenced and the sequences were analyzed. In a BLAST search, sequence shared 96% similarity towards the L segment of Qld-3432 and Ph isolates of Australia and Taiwan respectively. On the basis of the sequences in NCBI database having maximum identity (Acc. KC953852) towards the virus isolates of the present study, new set of primer pair (GKCaCVF/R) was designed from the S RNA (nt 2371-3608) in such a way to cover complete nucleocapsid gene (830 nt) of CaCV with an amplicon product 1237 bp size. Amplified product was cloned and sequenced (Figure 2) [12-14].

BLAST n analysis this of nucleotide sequence data of the complete coat protein gene of isolates revealed a maximum identity of 98% with CaCV isolates, EF625228 and KM014660 reported from chilli in India. In the sequence analysis, this isolate shared a maximum identity of 99.1% and 100% at nucleotide and amino acid level respectively towards the CaCV isolates reported from India (EF625228 and KM014660). Wide ranges of identities (86.4 to 99.1%) were observed among the CaCV isolates taken under the study. CaCV isolates from Taiwan and China were found to be highly divergent (DQ355974 and HM021139) sharing 86.4% identity and the remaining isolates shared 95.4 - 99.1% identity. In phylogenetic analyses, this isolate forms a separate group with the CaCV isolates reported from *Capsicum annum* from Aurangabad and Tamil Nadu of India rather than the isolate reported from Tomato India (Figure 3). This shows that, isolate under the present study had same center of origin as that of isolates reported earlier from India.

Host range study

The ELISA and RT- PCR positive samples of chilli were used to transmit the virus to indicator hosts by mechanical sap inoculation (Table 1). The virus produced concentric chlorotic spots in *Vigna*

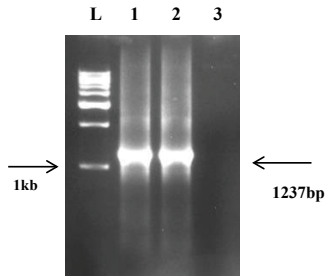

Figure 2: Agarose gel electrophoresis showing amplification of CaCV using specific primer pairs GK CaCV F1/R1.

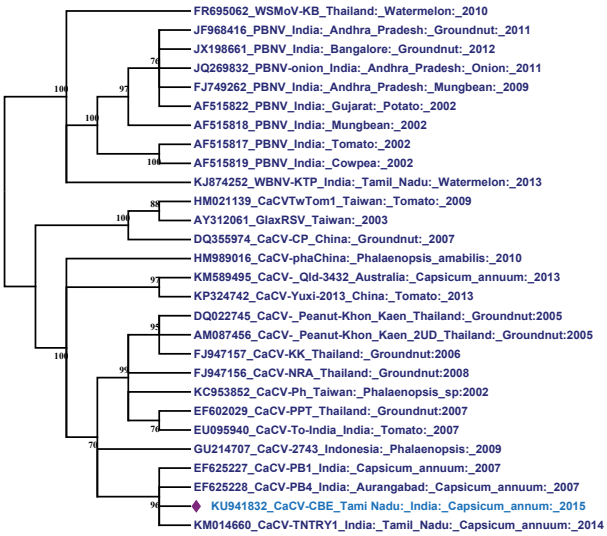

Figure 3: Phylogenetic dendrogram based on the alignment of complete nucleotide sequence of Nucleocapsid gene of CaCVCBE with that of nucleocapsidgene of selected tospoviruses.Values at notes represent the percentage bootstrap scores (1000 replicates) only values more than 70 are shown.

Table 1: Reaction of CaCV on different host plants through mechanical sap inoculation; Dpi – Days post inoculation; NI – Not infected.

Plant species	Family	Day taken for symptom expression	symptoms	Local/ systemic
Vigna unguiculata L.	Fabaceae	3-4 days	Concentric chlorotic spots	Local and systemic
		9th day	Necrotic spots	
Nicotiana tobacum L	Solanaceae	9-10 days	mosaic, veinal necrosis	systemic
		20th day		
Chenopodium amaranticolor L	Amaranthaceae	8th day	Chlorotic spots	local
Trianthema portulacastrum	Aizoaceae	4- 5days	Necrotic spots	Local
Capsicum annum L	Solanaceae	8-9 days	Concentric necrotic rings	local
Amaranthus viridis L	Amaranthaceae	6th day	Chlorotic lesions	local
		10th day	Necrotic lesions	
Arachis hypogea L	Leguminaceae	5-6 days	Chlorotic lesions	local
Glycine max L	Fabaceae	6-7th day	Chlorotic spots and necrosis	Local and systemic
Boerhavia diffusa L	Nyctaginaceae	6-7th day	Reddish spots and lesions	local
Vinca rosea	Apocynaceae	6-7th day	necrotic spots and lesions	local and systemic
Lagenaria siceraria L	Cucurbitaceae	NI	-	-
Cucurbita moschata L	Cucurbitaceae	NI	-	-
Cucumis sativus L	Cucurbitaceae	NI	-	-
Benincasa hispida	Cucurbitaceae	NI	-	-
Momordica charantia L	Cucurbitaceae	NI	-	-

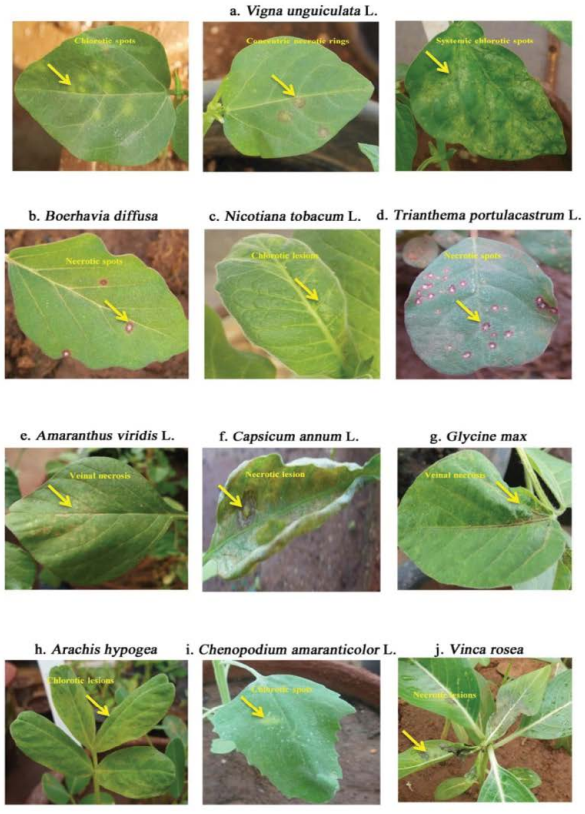

Figure 4: Reaction of CaCV on the different host plants through mechanical sap inoculation.

unguiculata at 3–4 dpi and necrotic spots at 9th dpi, later systemic infection resulted on the leaves at 15th dpi after inoculation. Systemic mosaic symptoms in *Nicotiana tobacum* were observed at 9–10 dpi, which subsequently led to veinal necrosis at 20 dpi. A minimum of 4 - 5 days were required to produce local lesions (chlorotic and necrotic) of CaCV on the host plants of *Trianthema portulacastrum* and *Arachis hypogea*, 6-7 days on *Amaranthus viridis* L, *Glycine max* and *Boerhavia diffusa,* 8-9 days on *Chenopodium amaranticolor* L and *Capsicum annuumL*. and 10-12 days on *Nicotiana tabacum* L. Systemic necrotic spots and lesions were observed on *vincarosea* at 6-7 DPI. The DAS-ELISA analyses of these samples were positive. There was no visible symptom expression on the plants of *Cucurbita moschata*, *Cucumis sativus, Benincasa hispida, Momordica charantia and Lagnearia siceraria L* Figure 4. The DAS-ELISA analysis of these samples resulted in negative results. Chen [15] tested 31 plant species out of which 18 were susceptible to CaCV-CP. The virus systemically infected *Arachis hypogaea*, *Phaseolus vulgaris*, *Glycine max, Pisumsativum, Phaseolus mungo, Cassia tora, Sesbaniacannabina, Nicotiana tobacum, N. rustica, N. glutinosa, N.occidentalis, N. benthamiana, Lycopersicone sculentum, Physalisfloridama, Petunia hybrida, Datura stramonium, Capsicum annuum* and *Cyamopsis tetragonoloba.* They have observed that symptoms on these hosts were mostly ring or yellow spots and necrosis on leaves. Local chlorotic ring spots and necrotic spots were found on inoculated leaves of *Chenopodium amaraticolor*, *C. quinoa, Gomphrenag lobosa, Vigna unquiculata, V. unguiculata ssp. sesquipedalis* and *Cassia occidentalis*. Seven plant species (*Viciafaba, Cajanuscajan, Catharanthu sroseus, Cicer arietinum, Zinnia elegans, Cucumissativusand Sesanumindicum*) were not infected. Zheng [16] also determined a host range of CaCV isolate, 91-orchid-1 and found that out of the twenty-three plant species mechanically inoculated, fifteen species were susceptible to the virus 91-orchid-1. Chlorotic local lesions were found on inoculated leaves of *N. glutinosa*, *N. occidentalis, N. tabacum* cv. Hicks, *N. tabacum var*. Samsun, *C. quinoa* and *C. murale*. Systemic infection of 91- orchid-1 was observed 6–10 days post-inoculation in *N. benthamiana, N. edwardsonii, N. tabacum var. Xanthi, N. tabacum cv. Vam-Hicks, N. rustica, Lycopersicone sculentum, Capsicum annuum* (red pepper), C. *annuum var. grossum* (sweet pepper) and D. *stramonium*. Symptoms on systemic hosts were mostly chlorotic rings pots or chlorotic spots initially which later developed into necrosis in the central region of spots. Infections were

also confirmed by positive reactions of ELISA tests with antisera to 91-orchid-1. Cucurbita pepo var. zucchini, *Cucumis melo* (muskmelon), C. *melo var. conomon Makino* (oriental pickling melon), *Citrullus vulgaris* (watermelon), *Luffa acutangula*(loofah), C. *melo var. makuwa* Makino (melon) and *Lagenaria siceraria* (bottle gourd) were not infected with 91-orchid-1 as determined by symptom expression, ELISA and infectivity assay on *C. quinoa*. In a host-range study, out of 25 plant species tested, 19 were susceptible to CaCV-Ch-Pan and CaCV To-Ind (*Capsicum chlorosis* virus-Tomato-India). Local and systemic infections were confirmed by observing symptoms on inoculated and newly emerged leaves. The infected leaves were tested for virus by DAS-ELISA using PAbs specific for the CaCV N protein [17].

From this study, it is evident that CaCV is the causal agent for the necrosis and chlorosis spot diseases of chilli and it is found spreading in Tamil Nadu. In future, this may cause menace for the cultivation of chilli crop. Hence, this preliminary work will be helpful for the specific identification of *Tospovirus* species, CaCV associated with the chilli crop.

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