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Current status of *Citrus tristeza virus* incidence and its spatial distribution in citrus growing geographical zones of India

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ABSTRACT

Citrus tristeza virus (CTV), a member of genus *Closterovirus*, is an important pathogen which has destroyed more than one million citrus trees in India till date. Survey was conducted in commercial *Citrus* sp. covering all the citrus growing geographical zones of India. For detection of CTV and estimation of disease incidence, direct antigen coated-enzyme linked immuno-sorbent assay (DAC-ELISA), reverse transcription- polymerase chain reaction and nucleotide sequence analyses were employed. The overall CTV incidence of 26.3% in Vidarbha region, 47.1-56.0% in Northeast (Asom, Meghalaya, Sikkim and the Darjeeling hills), 36-50% in South (Andhra Pradesh and Karnataka) and 16-60% in North-Northwest (Uttarakhand, Delhi and Punjab) were reported. CTV titre in infected tree samples of different locations was measured by ELISA . The high titre was observed in Kagzilime (*C. aurantifolia*) and sweet orange (*C. sinensis*) but low in mandarin (*C. reticulata*). Nucleotide sequence of 5'ORF1a fragment of CTV genome of nine representative CTV isolates, Kat-8, RA-3, B-6, Dh-1, An-9, TP-4, Pant-3, MU-2 and As-2 from all the citrus growing regions surveyed were analysed. The present Indian isolates showed 78-98% identities among them, and placed in different clusters in phylogenetic analysis. This is for the first time an overall incidence of CTV and its spatial distribution in citrus growing geographical zones of India were reported. Findings presented in this study will help in understanding epidemiology and formulating molecular based management strategy.

Key words : *Citrus tristeza virus*, Disease incidence, Distribution, Genetic diversity

Citrus tristeza virus (CTV), a member of genus *Closterovirus*, is an important viral pathogen which has killed about 100 million citrus trees worldwide over last 70 years (Bar-Joseph and Dawson 2008). The virus contains a long flexuous particle of 2000 × 10-12 nm size, transmitted by brown citrus aphid (*Toxoptera citricida*) predominantly in a semi-persistent manner (Bar-Joseph and Dawson 2008). CTV genome contains a single-stranded positive strand RNA molecule of 20 kb length, comprising 12 open reading frames (Karasev *et al.* 1995). CTV causes various disease symptoms like mild symptoms to seedling yellows, stem pitting, severe stunting and ultimately decline of the plant depending on citrus species, virus strains, scion/rootstock combinations and environmental factors (Lee and Bar-Joseph 2000).

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Citrus is cultivated in all the four geographical zones of India; Northeast, Northwest, Central and South, occupying 9.87 lakh ha with annual 96.38 lakh tonnes fruit production (Anonymous 2010). India produces different kinds of mandarins (*Citrus reticulata*), sweet orange (*C. sinensis*) and acid lime (*C. aurantifolia*), sweet lime (*C. limettoides*) and lemon (*C. lemon*) commercially. CTV occurs in nearly all the commercial citrus species in India (Ahlawat 1997, Biswas 2008, Biswas 2010, Kishore *et al.* 2010, Sharma *et al.* 2011).

Diagnosis of CTV in field condition is difficult as infected trees do not necessarily produce characteristic symptoms. Earlier, CTV was detected based on symptoms produced in indicator host, Kagzilime (*C. aurantifolia*) in India. However, development of symptoms in indicator hosts depends on environmental temperature and virus strains (Ahlawat and Pant 2003, Biswas 2008, Sharma *et al.* 2011). Therefore, enzyme linked immuno-sorbent assay (ELISA) (Biswas 2008, Chakroborty *et al.* 1992, Kishore *et al.* 2010), reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing (Biswas 2008, Sharma *et al.* 2011) have been used earlier to detect the virus.

Occurrence of CTV is common in mostly all the citrus-growing geographical zones of India, however overall disease

incidence in India was not reported earlier. Therefore, an attempt was made to study incidence and distribution of CTV in India using direct antigen coated (DAC)-ELISA, RT-PCR and nucleotide sequencing.

MATERIALS AND METHODS

Surveys were conducted in commercial *Citrus* species covering all the citrus growing zones of India. In Northeast and East, sweet orange Khasi mandarin (*C. reticulata*), Assam lemon (*C. lemon*), Kagzilime (*C. aurantifolia*) and pumello (*C. paradisi*) orchards from different areas of Asom and Meghalaya, and Kagzilime orchards in Kalyani area of West Bengal were surveyed (Fig. 1). Orchards in Citrus Research Station (CRS) of Assam Agricultural University at Tinsukia, Asom and Kagzilime orchard in Citrus Farm of Bidhan Chandra Krishi Viswavidyalaya (BCKV) at Kalyani, West Bengal were surveyed.

Nagpur mandarin (*C. reticulata*), sweet orange, Kagzilime and sweet lime orchards from different areas of Vidarbha region of Central India were surveyed (Fig 1). In South India, Kagzilime, sweet orange and sweet lime orchards in different areas of Karnataka and Andhra Pradesh were surveyed. Further in Northwest, Kinnow mandarin (*C. reticulata*) orchard from Abohar and Ferozepur areas of Punjab, and Sriganganagar area of Rajasthan were surveyed. Citrus orchards from four to eight villages under each of the areas were surveyed. Two to three orchards from each village, and 5-10 trees from each orchard were selected for collection of samples. Two twigs from each of the selected trees were brought to laboratory for diagnostic assay.

About 500 mg of citrus samples containing mixture of tender bark and mid veins were ground to fine powder using liquid N_2. Samples were assayed to detect CTV using DAC-ELISA (Biswas 2008). ELISA reaction was measured at 405 nm in Sunrise Tecan ELISA reader. Samples were considered CTV positive in ELISA if the absorbance reading at 405 was 2-3 times or more of the absorbance values of the healthy control. Disease incidence (% plant infection) was estimated using standard method: number of samples infected divided by number of samples tested and multiplied by 100.

Total plant RNA was extracted from 30 mg tender bark tissues using SV Total RNA isolation system kit (Promega, Madison, USA) and the first strand cDNA was synthesized using M-MLV-Reverse transcriptase (Promega, Madison, USA) following manufacture's instructions. The RT-PCR assay was performed, using primer pair (forward KLM 488 and reverse KLM 491) for amplification of 404 nt fragment from 5'ORF1a (positioned 1076 to 1479 nt) region of CTV genome (Biswas 2010).

Nine ELISA positive CTV samples were selected randomly and designated as isolate Kat-8 from Katol, RA-3 from Raichur, B-6 from Bangalore, Dh-1 from Dharwad, An-9 from Anantapur, TP-4 from Tirupati, Pant-3 from Pantnagar, MU-2 from Umsaitsting, Meghalaya and As-2 from Tinsukia, Asom. The PCR products obtained using primers KLM 488 and KLM 491 were purified and cloned into T&A vector (RBC, UK) (Biswas 2010). For each isolate, two clones were sequenced using the vector derived primer M13F in AB 13130 Genetic analyzer (Chromous Biotech, Bangalore). The consensus sequences were used for sequence analysis.

The sequence alignments were carried out using Clustal X version 1.81 (Thomson *et al.* 1997) and sequence identities were compared using GeneDoc version 2.6.002. Nieghbour-joining phylogenetic tree was constructed using the program MEGA4 (Tamura *et al.* 2007). Corresponding sequences of previously reported distinct Indian CTV isolates, Kpg1, Kpg2, BAN-1 and BAN-2 (Biswas 2010; Roy *et al.* 2005) and six representative CTV genotypes VT, T36, T30, B165, NZ-RB and HA16-5 recognized internationally (Melzer *et al.* 2010, Roy and Brlansky 2010, Biswas *et al.* 2011) were used for sequence analysis and comparison.

RESULTS AND DISCUSSION

Disease diagnosis and disease incidence

Infection of CTV in citrus orchards surveyed in the present study was detected through ELISA and RT-PCR. Virus infection was observed in majority of mandarin orchards in Vidarbha region of Central India, however, percent infection varied from orchard to orchard. Disease infection was detected in Mudkhed mandarin orchard in Katol, sweet lime and Tahiti lime orchards in Akola of Vidarbha region. All the Kagzilime and sweet orange orchards surveyed were infected by CTV, however, disease incidence varied depending on orchards. Overall CTV incidence in Vidarbha region was as high as 41% in sweet orange, 28% in mandarins and as low as 13% in acid lime.

In Northeast, all the Kagzilime, and Assam lemon and majority of Khasi mandarin orchards surveyed in Asom were found to be CTV infected. The disease incidence varied from 22 to 71% in different citrus species at different areas. The pumello orchards of Dhulijan area were found to be infected by this virus. All the Khasi mandarin, sweet orange, Assam lemon and Kagzilime orchards surveyed in Meghalaya were also infected by CTV showing disease incidence of 23-100%. The Kagzilime orchards surveyed in Kalyani areas of West Bengal in East, did not show CTV infection.

The Kagzilime, sweet orange and sweet lime orchards surveyed in South India were found to be CTV infected. In Karnataka, disease incidence was as high as 72% in sweet lime and 40% in sweet orange, whereas in Andhra Pradesh it was up to 80% in sweet orange and 32% and 7% in sweet lime and Kagzilime, respectively. Unlike Northeast, the pumello orchards in South India surveyed was not found to be CTV infected. Kinnow mandarin orchards surveyed in Punjab and Rajasthan of Northwest were tested but CTV infection was not detected in any sample of these areas.

Table 1 Overall disease incidence of CTV in different citrus growing geographical zones of India

Zone	Region	Over all incidence*
Central	Vidarbha	26.3
South	Andhra Pradesh	36.0
	Karnataka	50.0
Northeast	Assam	56.0
	Meghalaya	47.1
	Darjeeling hills[1]	48.2
	Sikkim[2]	46.2
North	Delhi[3]	20.0
	Pantnagar[4]	16-20
Northwest	Punjab[5]	>60

*Average of incidence of different citrus species and area in the particular region. [1]Biswas 2008, [2]Kishore *et al.* 2010, [3]Sharma *et al.* 2011, [4]Singh 2011, [5]Thind *et al.* 2005.

CTV infection was confirmed by RT-PCR using virus specific primers. The primer pair (KLM 488 and KLM 491) amplified desired sizes of 404 nt fragment from 5'ORF1a region of CTV genome from the citrus sample which were ELISA positive. The PCR amplification was not found from any of the ELISA negative citrus samples. Therefore, the RT-PCR confirmed the ELISA results which have been used to estimate disease incidence in the present study.

For the first time, overall CTV incidence in commercial citrus species grown in India was reported; showing 26.3-60% incidence (Table 1). Visible symptoms caused by CTV was not observed in mandarin and sweet orange under field conditions during surveys that supported the previous report (Chakrabarty *et al.* 1992, Biswas 2008, Kishore *et al.* 2010, Sharma *et al.* 2011). In acid lime and sweet lime characteristic vein clearing and vein flecking were observed in majority of the orchards surveyed. The present and previous studies indicated that sweet orange and acid lime was more susceptible than other commercial citrus species.

Distribution of CTV in Vidharva regions was reported earlier (Ghosh *et al.* 2003), however, disease incidence was not reported from this region. Present study reported incidence up to 41% in sweet orange, 22-28% in mandarins and 8-13% in acid lime of Vidarbha region with an overall incidence of 26.3%. Occurrence of CTV in South India was reported earlier (Ahlawat 1997) but no report of disease incidence was available. The present study reported an overall CTV incidence of 36% in Andhra Pradesh and 50% in Karnataka of South India.

Prevalence of CTV in Asom and Meghalaya was reported earlier (Ahlawat *et al.* 1992), however incidence and distribution of the virus were not studied. Present study showed that most of the commercial citrus species in Asom and Meghalaya were infected by CTV showing an overall disease incidence of 47-56%. In the Darjeeling hills and Sikkim hills, CTV incidence of 48.2% and 46.2% respectively, were reported earlier (Biswas 2008, Kishore *et al.* 2010). CTV incidence of 20% in Delhi (Sharma *et al.* 2011) and 16-20% in Pantnagar, a Terai region of Uttarakhand was estimated earlier (Singh 2011). In the present study, the virus was not detected in Kinnow mandarins surveyed in Punjab and Rajasthan, however, CTV incidence up to 60% from this area has been reported earlier (Thind *et al.* 2005). Kagzilime orchards of Kalyani areas of from West Bengal were tested, but CTV was not detected indicating absence of CTV in this areas.

The pumello is susceptible to CTV, but not in all the citrus-growing regions of India. This citrus species is infected in the Darjeeling hills (Biswas 2008) and Asom (present study), but not in Delhi (Sharma *et al.* 2011) and Bangalore (present study), which attribute to the differences in environments and virus strains prevailing in different regions, that might have played important role in determining susceptibility of host.

Virus titre

CTV titre in infected citrus was measured using absorbance values at 405 nm in ELISA reader. The titre values in different citrus species at different region were estimated. The overall titre value of 3.0 to 3.8 fold in Vidarbha, 2.5 to 5.6 fold in South, 3.1 to 11.9 fold in Northeast and 3.8 to 5.6 fold in North, were recorded. The sweet orange and Kagzilime recorded high titre value up to 11.9 fold in Kagzilime and 8.8 fold in sweet orange (Table 2).

The variation of CTV titre in citrus species in different

Table 2 Estimation of virus titre in CTV infected citrus trees

Zone	Area	Virus titre in citrus host					
		Kagzilime	Mandarin	Pumello	Sweet orange	Assam lemon	Sweet lime
Central	Vidarbha	0.92 (3.1)	0.9 (3)		0.91 (3)		1.16 (3.86)
South	Andhra Pradesh	1.05 (3.0)			1.97 (5.63)		0.9 (2.57)
	Karnataka				1.4 (4.0)		1.12 (3.2)
Northeast	Assam-Meghalaya	2.23 (4.05)	1.93 (3.50)	1.7 (3.1)	3.0 (5.45)	2.93 (5.32)	
	Darjeeling[1]	3.1 (11.9)	1.8 (6.9)	1.8 (6.9)	2.3 (8.8)		
North	Delhi[2]	1.68 (5.6)	1.16 (3.86)		1.64 (5.46)		

Data in parenthesis denote the X fold of virus titre in infected trees compared to OD values of healthy control of: 0.3 (Central), South 0.35 (South), 0.55 (Northeast) and 0.30 (North); [1]Biswas (2008), [2]Sharma *et al*. (2011).

regions was observed in the present study that might be attributed to: (i) varied degrees of virus multiplication under different environmental conditions, (ii) occurrence of diversified CTV isolates and (iii) different hosts exhibiting differences in resistance/susceptibility in particular region. Low titre value was observed in mandarins, although mandarin decline due to CTV infection is common (Biswas 2008, Biswas *et al.* 2011), suggesting concentration of CTV in plant is not the only factor for causing disease severity in citrus.

Sequence analysis and genetic diversity of CTV

The 5'ORF1a fragment (404 nt) from nine CTV isolates were cloned, sequenced and deposited in GenBank (Fig 1). Sequence analysis showed that the present CTV isolates had 83-98% identities among them and 77-97% identities with other CTV isolates (Table 3). The isolate Pant-3 (North) was closely related with B-6 (South) by 98% identity and isolate An-9 (South) closely related to Northeast isolates As-2, MU-2, Kpg1; Delhi isolate D1 and Vidarbha isolate Kat-8 by 94-97%. Among South isolates An-9, TP-4, Dh-1, B6, BAN-1, BAN-2 and B165 the sequence identity ranged from 84 to 91%. Assam isolate As-2 and Meghalaya isolate MU-2 had 91% identity between them. Dharwad isolate Dh-1 was closely related to Hawaii isolate HA16-5 by 95% identity (data not shown).

The phylogenetic analyses using present and other CTV isolates generated overall seven clades. The present isolates placed in six clades. Isolates Pant-3, B-6, TP-4 and previously reported Indian isolate Kpg2, all belonging to different geographical regions, grouped together with Florida mild isolate T30 (Fig 1). However, Kat-8, D1, RA-3, Kpg1 and An-9, all belonging to different regions, grouped together along with Israel severe isolate VT. The Dharwad isolate Dh-1 grouped with Hawaii CTV isolate HA16-5. The Asom isolate As-2, Meghalaya isolate MU-2 and Bangalore isolate BAN-1 all segregated as distinct entity in the phylogenetic tree.

The sequence analyses demonstrated the occurrence of extensive genetic diversity among the Indian CTV isolates that supported the diversity at global level as reported earlier (Hilf *et al.* 2005, Roy *et al.* 2005, Biswas 2010, Sharma *et al.* 2011, Rubio *et al.* 2001). Indian CTV isolates of different citrus-growing regions clustered together, suggesting that phylogenetic clustering of CTV isolates do not reflect their geographical origin as reported earlier (Rubio *et al.* 2001, Martin *et al.* 2009). This could be due to (i) traffic of infected propagative material or (ii) recombination among diverged sequences. Earlier, recombination events in CTV isolates were reported from India (Biswas *et al.* 2011, Sharma *et al.* 2011) and other countries (Martin *et al.* 2009, Roy and Brlansky 2010), which provides the support for emergence of genetic variants in different geographical zones.

Estimation of disease incidence and distribution of virus and its variants reported in the present study will help in

Table 3 Pair-wise percent nucleotide identities of 5'ORFa fragment of Indian and other CTV isolates

	Indian isolates													Representative genotype						
	An-9	As-2	B-6	Dh-1	Kat-8	MU-2	Pant-3	RA-3	TP-4	BAN-1	BAN-2	D-1	Kpg1	Kpg2	B165	HA16-5	NZRB-G90	T30	T36	VT
An-9		96	92	85	97	94	92	91	91	93	84	97	97	92	85	86	83	84	82	96
As-2			90	85	95	91	90	90	91	92	83	95	95	90	84	87	83	82	81	93
B-6				84	90	89	98	89	90	89	81	90	90	92	86	86	80	83	80	88
Dh-1					84	83	84	84	84	83	79	84	85	84	88	95	79	77	78	84
Kat-8						93	90	90	89	92	83	96	96	90	84	85	82	82	81	94
MU-2							88	88	87	89	81	92	93	88	87	86	82	81	79	92
Pant-3								89	90	89	81	90	90	92	85	86	80	83	80	88
RA-3									87	88	82	90	91	88	83	86	81	81	80	89
TP-4										88	81	89	89	90	83	86	81	83	80	88
BAN-1											83	92	92	89	84	85	82	81	81	90
BAN-2												83	83	83	82	81	96	75	91	81
D-1													96	90	84	85	82	82	81	94
Kpg1														90	85	87	83	83	82	95
Kpg2															85	86	83	89	82	89
B165																89	82	77	80	83
HA16-5																	82	77	82	85
NZRB-G90																		74	91	81
T30																			74	82
T36																				80
VT																				

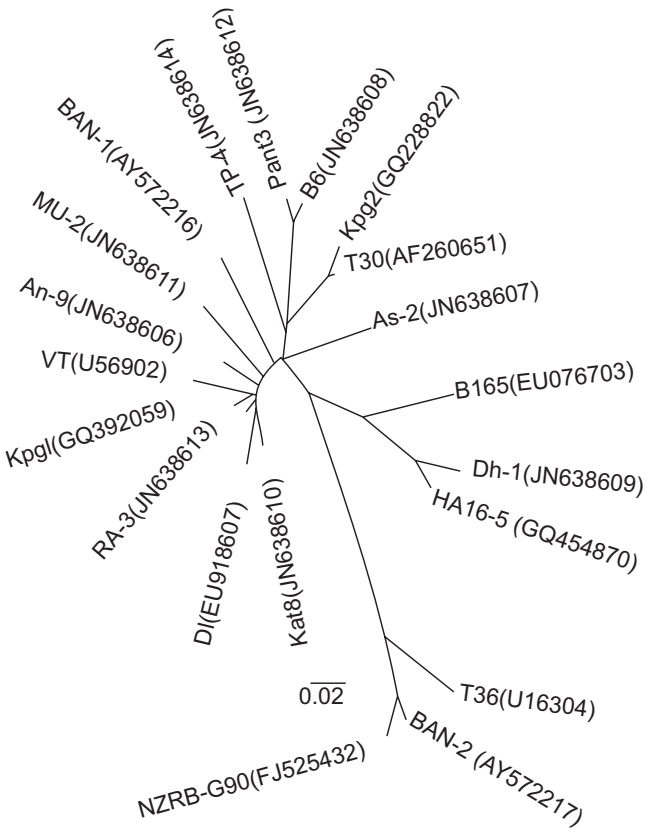

Fig 1 Phylogenetic relationships of present Indian isolates with previously reported Indian and international CTV isolates using maximum likelihood method in MEGA4. Scale bar represent the genetic distance, proportional to the number of nucleotide differences between branch nodes. Statistical significance was estimated performing 100

understanding epidemiology and formulating molecular based management strategy of the disease. For the first time, effort has been made, to report overall or zone-wise disease incidence in India. Use of planting materials without certification programme is the main reason for dissemination spread of CTV and its variants, therefore, clean cultivation using sensitive molecular diagnostics and supply of disease free planting materials are effective control measures of CTV, which will make Indian citrus industries more profitable.

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