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Present status of *Citrus tristeza virus* infecting *Citrus* spp. in Darjeeling hills and its detection in different plant parts

Amalendu Ghosh · Amrita Das · K. B. Pun · R. Kumar · Ramu Meena · V. K. Baranwal

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Abstract Darjeeling hills of West Bengal (India) are well known for the production of mandarin orange (*Citrus reticulata*). The spread of *Citrus tristeza virus* (CTV) in this area has threatened the cultivation of citrus. Therefore, a study of the incidence and distribution of CTV in Darjeeling hills was undertaken and a geographical map was prepared. In DAS-ELISA, a high titre of CTV was recorded in the tender shoot bark, leaf petiole and mid-rib of seven *Citrus* spp. Three citrus species, *viz*., pomelo, trifoliate and kumquat, were found to be free of CTV infection when tested in double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription polymerase chain reaction (RT-PCR).

Keywords *Citrus reticulata* · Distribution · India · Mandarin orange · *Tristeza* · CTV · Darjeeling

A. Ghosh · A. Das (✉) · K. B. Pun · R. Kumar · R. Meena
Indian Agricultural Research Institute, Regional Station, Kalimpong 734 301 West Bengal, India
e-mail: amritapatho@gmail.com

V. K. Baranwal
Advanced Centre for Plant Virology, Indian Agricultural Research Institute,
New Delhi 110 012, India

Present Address:
K. B. Pun
Regional Rainfed Lowland Rice Research Station, Central Rice Research Institute,
Gerua 781 102 Assam, India

Present Address:
R. Kumar
Indian Agricultural Research Institute, Regional Station,
Karnal 132 001 Haryana, India

Introduction

Citrus tristeza virus (CTV) is an economically important virus in all citrus-growing areas throughout the world, destroying millions of citrus trees over the last 70 years (Bar-Joseph & Dawson, 2008). CTV belongs to the *Closterovirus* genus of family *Closteroviridae*. It is a phloem-limited virus. The genome consists of a positive sense single strand RNA comprising 19,296 nucleotides and organized into 12 open-reading frames (Bar-Joseph *et al*. 1989; Karasev *et al*. 1995; Ruiz-Ruiz *et al*. 2007). Darjeeling hills of West Bengal are well known for the production of mandarin orange (*Citrus reticulata* Blanco), which fetches high export earnings to citrus growers. However, CTV causes severe loss in mandarin production in Darjeeling hills (Biswas *et al*. 2004; Mukhopadhyay *et al*. 1986). Earlier molecular diagnosis of CTV and sequencing of the complete genome of the Kpg3 isolate were reported from this region (Biswas 2008; Biswas *et al*. 2012), but no definite information on incidence and distribution of CTV in this area was available. In this work, we studied the distribution of CTV in mandarin orchards of Darjeeling hills. Effort was also expended to determine the best tissue type for detection of CTV through double antibody sandwich–enzyme linked immunosorbent assay (DAS-ELISA) and CTV-free *Citrus* spp.

Materials and methods

Citrus-producing areas of Darjeeling hills were surveyed during the winter season of 2011–2013. Mandarin plants 12–15 years old in different orchards were selected randomly for collection of leaf and bark samples. A total of

554 samples from 25 locations were collected during the field survey. Samples were packed in plastic bags, taken to the laboratory and processed immediately. Unused samples were stored at –80°C for future use.

DAS-ELISA was performed for CTV detection (Clark and Adams 1977) in all the samples using polyclonal antiserum and respective conjugate (Bioreba) and the OD values were taken at 30 min after addition of substrate. OD values at least three times higher than the negative control were taken as positive for CTV infection. Samples were rated as high, moderate, low and healthy based on their relative OD values. Percent CTV infection was determined by calculating the number of plants infected with CTV among the total number of plants sampled. All the places surveyed were plotted on the geographical map of Darjeeling hills. The places of low, medium and high CTV titre in citrus as well as healthy citrus were marked on the map based on the average OD value.

Darjeeling mandarin (5, 10 and 15 years old) along with rough lemon, pomelo, Assamese lime, kagzi, Rangpur lime, sour orange, mosambi, kumquat, trifoliate (8–10 years old) and CTV-infected mandarin plants graft-inoculated on rough lemon, kagzi lime and mosambi (5 years old) from the IARI, Kalimpong Research Farm, were used to determine CTV antigen concentration by DAS-ELISA in different parts of the plants, *viz*., mid-rib of leaves, leaf lamina, petiole, thorn, tender shoot bark, feeder root, fruit peel and fruit stalk.

Table 1 Incidence and distribution of CTV in mandarin orchards of Darjeeling hills

Location of orchard	Average OD (SEM)	-Fold change over –ve control	% Infection (n)[y]
St Joseph, Kalimpong	1.523 (0.0326)[z]	12	[x]100.00[a] (23)
Peshok	1.086 (0.251)	9	63.64[b] (11)
Bijanbari-1	1.485 (0.046)	12	100.00[a] (22)
Bijanbari-2	1.187 (0.099)	10	87.50[a] (24)
Mirik-1	1.774 (0.138)	14	90.91[a] (22)
Mirik-2	1.620 (0.154)	13	88.24[a] (17)
Mirik-3	1.533 (0.044)	12	100.00[a] (17)
Latpanchar	0.146 (0.008)	1	0.00[e] (3)
UBKV, Kalimpong	0.874 (0.140)	7	90.91[a] (11)
Pedong-1	1.148 (0.042)	9	100.00[a] (6)
Pedong-2	0.933 (0.050)	8	100.00[a] (11)
Takling	0.847 (0.083)	7	100.00[a] (6)
Bhage	0.748 (0.066)	6	89.47[a] (19)
Yagdhe	0.649 (0.068)	5	75.00[b] (24)
Bingbong	0.707 (0.077)	6	79.17[b] (24)
Mahakaldara	0.644 (0.051)	5	100.00[a] (19)
Mansong	0.677 (0.275)	6	50.00[c] (4)
Mangmaya	0.454 (0.025)	4	100.00[a] (10)
Posher basty	0.229 (0.037)	2	33.33[d] (9)
Kamjer	0.363 (0.022)	3	100.00[a] (8)
Tista valley	1.089 (0.085)	9	100.00[a] (10)
Kashyem	0.615 (0.025)	5	66.67[b] (6)
CDRS, Kalimpong	0.842 (0.056)	7	50.00[c] (10)
Jholung	0.845 (0.056)	7	50.00[c] (12)
IARI, Kalimpong Research Farm	1.395 (0.134)	11	86.46[a] (226)
-ve control	0.123		
+ve control	1.355		

[z]Values in parentheses are SEM values

[y]n=sample size

[x]Within the column, values followed by a common letter do not differ significantly from each other at P=0.05

Reverse transcription–polymerase chain reaction (RT-PCR) was performed to confirm the result of DAS-ELISA assay in samples of Darjeeling mandarin, pomelo, trifoliate and kumquat. Total RNA was extracted from 100 mg of shoot bark, midrib and petiole from representative samples using RNeasy Plant Mini kit (Qiagen, Alameda, CA, USA) following the manufacturer's protocol. The following forward and reverse primers were used for the amplification (588 bp) of the major coat protein gene of the CTV; 5′ ATGGACGACG AAACAAAGAAAT 3′ and 5′ AGCTTGTATGTACA CAGCAC 3′, respectively, by RT-PCR.

The first strand cDNA was synthesized using total RNA extracted from citrus tissues as templates and the reverse primer with Maxima first strand cDNA synthesis kit (Thermo Scientific, Rockville, MD, USA) according to the manufacturer's protocol. To amplify the major coat protein gene, polymerase chain reaction (PCR) was performed in 50 μl of reaction mixture containing 5 μl of cDNA, 0.2 mM of each dNTP, 10 μM of each primer, 1× PCR buffer and 1U of DNA polymerase (DreamTaq, Fermentas). The PCR cycling profile was consisted of one cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 56 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR products were separated in 1 % agarose gel in Tris-acetate (TAE) buffer along with GeneRuler 1 kb Plus DNA ladder.

Results

Of 554 Darjeeling mandarin trees, 467 trees were found positive for the CTV infection by DAS-ELISA (Table 1, Fig. 1). Eighty percent to 100 % incidence was recorded in orchards located at St. Joseph, Kalimpong; Bijanbari-1 & 2; Mirik-1, 2 & 3; UBKV, Kalimpong; Pedong-1 & 2; Takling; Bhage; Mahakaldara; Mangmaya; Kamjer; Teesta valley and IARI-Kalimpong Research Farm. Fifty percent to 79 % infection of CTV was observed in Peshok; Yagdhe; Bingbong; Mansong; Kashyem; CDRS, Kalimpong and Jholung. A low incidence (33.33 %) of CTV was noted in mandarin orchards of Posher basty. And the Mandarin orchard at Latpancher was found to be free of CTV infection. High concentration of CTV antigen (10–14-fold higher) was recorded in St. Joseph,

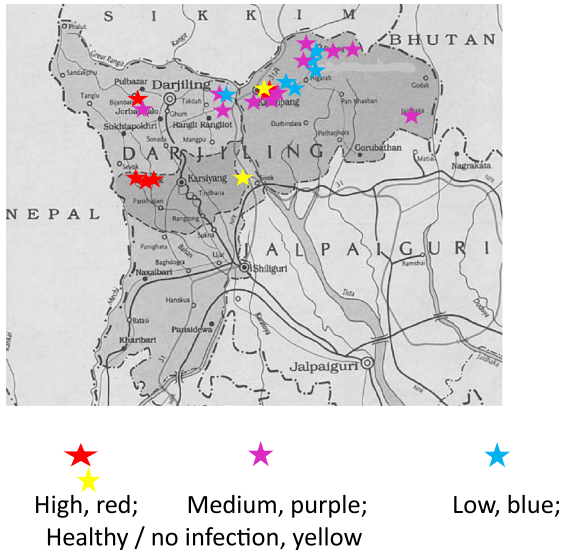

Fig. 1 Distribution of CTV in Darjeeling hills

Kalimpong; Bijanbari-1 & 2; Mirik-1, 2 & 3, IARI-Kalimpong Research Farm. A low average OD value in DAS-ELISA assay was recorded in the mandarin plants of Latpanchar and Posher basty.

The concentration of CTV antigen in different parts of infected citrus hosts on the basis of DAS-ELISA reaction varied significantly (Table 2, Fig. 2). Virus titre was scored on a four-scale rating as high (+++), medium (++), low (+), and no virus (−) titre. Virus titre was low in all parts of 5-year-old Darjeeling mandarin plant samples. Very high virus titre was noticed in tender shoot bark, mid-rib and leaf petiole of 10- and 15-year-old mandarin samples. CTV antigen titre was more in 8–10-year-old kagzi lime and Darjeeling mandarin followed by Assamese lime, Rangpur lime, rough lemon, mosambi and sour orange. A low titre of virus was noticed in sour orange. Among the 5-year-old graft-inoculated plants, Darjeeling mandarin grafted on rough lemon root stock had maximum virus titre compared with other root stocks. No reaction was observed in healthy Darjeeling mandarin, indicating that these reactions were CTV-specific. Pomelo, trifoliate and kumquat did not show positive reaction for CTV in any part of the plant. Absence of CTV in these three species of citrus was confirmed in RT-PCR when no amplification was observed (data not presented). High titre of CTV was detected in leaf petiole (4–14-fold), mid-rib of leaf (4–21-fold) and tender shoot bark (6–18-fold) of all *Citrus* spp. infected with CTV. In the case of leaf lamina, which consists of mainly parenchyma tissue,

Table 2 CTV concentration in different parts of *Citrus* spp. at IARI-Kalimpong Research Farm

Citrus spp.	Age of plant (yrs)	Virus titre (OD value of different plant parts)							
		Leaf lamina	Leaf petiole	Mid-rib of leaves	Thorn	Tender shoot bark	Fruit peel	Fruit stalk	Feeder root
Mandarin	5	0.217(2)$^{-}$	0.617 (5)$^{+}$	0.529 (4)$^{+}$	0.698 (6)$^{+}$	0.986 (8)$^{++}$	NA	0.719 (6)$^{+}$	0.302 (2)$^{-}$
Mandarin	10	0.617(5)$^{+}$	1.085 (9)$^{++}$	1.983 (16)$^{++}$	1.113 (9)$^{+}$	2.283 (18)$^{+++}$	0.561 (4)$^{+}$	0.867 (7)$^{+}$	0.327 (3)$^{+}$
Mandarin	15	0.883(7)$^{++}$	1.474 (12)$^{+++}$	2.373 (19)$^{+++}$	0.488 (4)$^{+}$	1.715 (14)$^{+++}$	0.151 (1)$^{-}$	0.908 (7)$^{++}$	0.252 (2)$^{-}$
Rough lemon	8	0.596(5)$^{+}$	1.208 (10)$^{++}$	1.678 (13)$^{+++}$	0.91 (7)$^{++}$	1.429 (11)$^{+++}$	NA	1.799 (14)$^{+++}$	0.135 (1)$^{-}$
Pomelo	10	0.198(2)$^{-}$	0.167 (1)$^{-}$	0.157 (1)$^{-}$	NA	0.154 (1)$^{-}$	0.162 (1)$^{-}$	0.178 (1)$^{-}$	0.134 (1)$^{-}$
Assamese lime	10	0.729(6)$^{+}$	1.785 (14)$^{+++}$	2.002 (16)$^{+++}$	1.573(12)$^{+++}$	1.509 (12)$^{+++}$	0.175 (1)$^{-}$	2.128 (17)$^{+++}$	0.378 (3)$^{+}$
Kagzi lime	8	0.599(5)$^{+}$	2.084(17)$^{+++}$	2.616(21)$^{+++}$	1.134(9)$^{++}$	2.285(18)$^{++}$	NA	NA	0.403 (3)$^{+}$
Rangpur lime	8	0.684(5)$^{+}$	1.331 (11)$^{+++}$	2.074 (16)$^{+++}$	1.09 (9)$^{++}$	2.11 (17)$^{+++}$	0.274 (2)$^{-}$	1.617 (13)$^{+++}$	0.435 (3)$^{+}$
Sour orange	10	0.197(2)$^{-}$	0.498 (4)$^{+}$	0.298 (2)$^{-}$	0.308 (2)$^{-}$	0.792 (6)$^{+}$	NA	0.290 (2)$^{-}$	0.171 (1)$^{-}$
Mosambi	10	0.681(5)$^{+}$	0.900 (7)$^{++}$	1.727 (14)$^{+++}$	0.605 (5)$^{+}$	1.35 (11)$^{+++}$	0.179 (1)$^{-}$	0.883 (7)$^{++}$	0.340 (3)$^{+}$
Kumquat	10	0.233(2)$^{-}$	0.224 (2)$^{-}$	0.275 (2)$^{-}$	0.199 (2)$^{-}$	0.225 (2)$^{-}$	NA	NA	0.195 (2)$^{-}$
Trifoliate	10	0.138(1)$^{-}$	0.165(1)$^{-}$	0.158(1)$^{-}$	0.199(2)$^{-}$	0.21(2)$^{-}$	NA	NA	0.184 (1)$^{-}$
Mandarin grafted on rough lemon	5	0.358(3)$^{+}$	1.063 (8)$^{++}$	1.508 (12)$^{+++}$	0.444 (4)$^{+}$	1.404 (11)$^{+++}$	0.152 (1)$^{-}$	0.805 (6)$^{+}$	0.403 (3)$^{+}$
Mandarin grafted on Kagzi	5	0.28(2)$^{-}$	1.28(10)$^{++}$	0.812 (6)$^{+}$	0.628 (5)$^{+}$	0.567 (4)$^{+}$	NA	NA	0.296 (2)$^{-}$
Mandarin grafted on mosambi	5	0.239(2)	1.331(11)$^{+++}$	0.539 (4)$^{+}$	0.331 (3)$^{+}$	1.205 (10)$^{++}$	0.170 (1)$^{-}$	1.379 (11)$^{+++}$	0.311 (2)$^{-}$

Control (+)=1.358; Control (-)=0.126; <3=healthy (-); 3-6=low (+); 7-10=medium (++); >10=high (+++); values within parenthesis are CTV titre in folds higher than –ve control

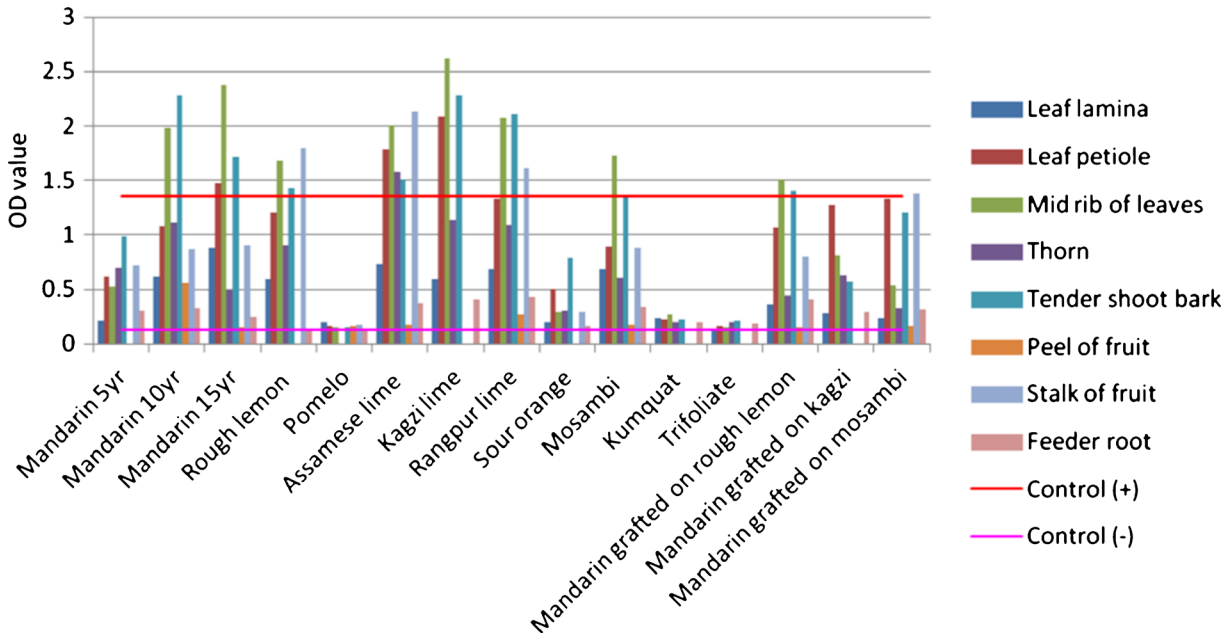

Fig. 2 Comparative titre of CTV in different parts of *Citrus* spp

low to medium antigen titre was recorded. Four–12-fold higher concentration of CTV was found in green thorn of different *Citrus* spp. CTV was not detected in the fruit peel of any of the *Citrus* spp. However, fruit stalk showed 6–17-fold higher titre. Assamese lime, rough lemon and Rangpur lime recorded a comparatively higher concentration (13–17-fold) of CTV in fruit stalk than other hosts. Underground plant parts, *i.e.*, feeder roots of all *Citrus* spp., showed a lower CTV antigen titre compared with above-ground parts.

Discussion

The presence of CTV in mandarin orchards of Darjeeling hills was reported previously by Mukhopadhyay *et al.* (1986) and Ahlawat *et al.* (1992). In the present study, spread of the virus was recorded in major citrus-growing areas of Darjeeling hills like Kalimpong, Bijanbari, Mirik, Pedong, Takling, Bhage, Mahakaldara, Mangmaya and Teesta valley. The low rate of infection noticed in areas like Latpanchar and Posher basty may have been due to some geographical isolation in hilly track or escape from vector feeding. The occurrence of CTV in some areas of Darjeeling during 2004–05 was reported by Biswas (2008). Earlier work in this area was focused on the symptomatology and characterization of the CTV. In the present study, more citrus-growing areas in Darjeeling hills were investigated which provided definite evidence of CTV spread in Darjeeling hills. CTV infection was recorded in rough lemon, Rangpur lime, Assamese lime and sour orange for the first time from this region.

The concentration of the antigen varies in different plant parts and also in different *Citrus* spp. (Brlansky *et al.* 1988). In the present study, a higher concentration of CTV was recorded in the older mandarin plants, which might be due to the increase of CTV concentration with age of the plant and multiple inoculations by aphids in older plants. In recent times, there is a demand for budded plants for quality fruits and yield. Although serological assays are available for detection of CTV, not much information is available for sampling of appropriate tissues for detection of CTV in the Darjeeling area. Our study indicated that leaf petiole, tender shoot bark and mid-rib of leaf with higher CTV titre can be used for CTV indexing. Low titre in the leaf lamina and fruit peel confirmed its phloem confining characteristics, as stated by Bar-Joseph *et al.* (1989). A thick crust of dead tissue in the periphery of root bark may contribute to the low titre of CTV. Pomelo, trifoliate and kumquat were found free of CTV infection even though they were exposed in the field for 10 years. This indicates that these plants might be either non-preferred hosts of vectors or resistant to CTV infection. General resistance

to CTV in trifoliate, kumquat and pomelo was reported by Fagoaga *et al*. (2005) and Ayazpour *et al*. (2011).

Thus, it is evident that CTV is widely distributed throughout the mandarin-growing areas of Darjeeling hills. Leaf petiole, tender shoot bark and mid-rib of leaf are the best tissue types for an indexing program to identify healthy mother plants and eradicate infected plants. This is the first time that an absence of CTV infection in pomelo, trifoliate and kumquat was found in India. However, more studies will be required to ascertain the resistance in these citrus species to different strains of CTV.

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