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Short communication

Distribution of *Citrus tristeza virus* in the Darjeeling hills and their biological symptoms in mandarin orchards

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ABSTRACT

Mandarin (*Citrus reticulata* Blanco), a traditional fruit crop growing at altitude from 500 to 1500 m in the Darjeeling hills of Northeastern Himalayan region, plays an pivotal role in the economy of the poor farmers of the region. *Citrus tristeza virus* (CTV), member of a phloem limited *Closterovirus* and transmitted by brown citrus aphid, is one of the important virus causing losses in mandarin orchards in the Darjeeling hills. A survey was conducted in mandarin orchards at 30 locations covering all the Taluks/blocks during April-July, 2011 and biological symptoms, incidence and distribution of CTV were studied. A general decline symptom along with chlorosis, poor as well as growth stunting was observed in majority of the mandarin orchards. ELISA showed that mandarin samples of 29 locations were CTV positive showing considerable virus titer (OD value) of 3-10 fold compared to healthy control. The ELISA positive samples were further confirmed by RT-PCR. The present study concluded that CTV is widely distributed in all the mandarin growing areas in the Darjeeling hills and CTV isolates occurring in this region are decline inducing isolates.

Key words: *Citrus tristeza virus*, *Citrus reticulata*, Darjeeling hills, ELISA, RT-PCR.

Mandarin (*Citrus reticulata* Blanco), a traditional fruit crop growing in the Darjeeling hills of Northeastern Himalayan region of India, plays an pivotal role in the economy of the poor farmers. Mandarin is cultivated in the area located at altitude from 500 to 1500 m asl, which receives an annual rainfall of 2500-3000 mm. Because of the poor investment in the cultivation and maintenance, and affect of several biotic and abiotic factors, the mandarin orchards are gradually being wiped out, resulting in drastic reduction of fruit production in this area (Mukhopadhyay *et al*., 12; Ahlawat and Raychaudhuri, 2; Biswas *et al*., 4). *Citrus tristeza virus* (CTV), a phloem limited, aphid (*Toxoptera citiricidus*) transmitted *Closterovirus*, is one of the important factors causing decline of mandarin in the Darjeeling hills (Ahlawat and Raychaudhuri, 2; Chakraborty *et al*., 11; Biswas, 4). CTV is a destructive virus causing decline and death of about 100 million citrus trees worldwide including more than one million trees in India (Bar-Joseph and Dawson, 3; Ahlawat, 1). CTV contains long flexuous filamentous particles of 2000 x 11 nm in dimension and ssRNA molecules of 19.3 kb size (Bar-Joseph and Dawson, 3).

The virus in this region has been characterized based on biological reaction and sequencing of viral genome, and several CTV variants have been reported from Northeast regions of India (Biswas, 4, 6; Biswas *et al*., 7, 8; Tarafdar *et al*., 13). Biological host range and complete genome analysis revealed that CTV in the Darjeeling hills is likely to be decline inducing virus (Biswas *et al*., 8). Although, occurrence of CTV has been reported and many virus isolates has been characterized, distribution of this disease in all the blocks (Taluk) under three sub-divisions, Kalimpong, Karseong and Darjeeling in the Darjeeling hills has not been determined properly. Therefore, effort has been made in the present study to for an extensive survey of mandarin orchards covering all the blocks in this hill regions region and subsequently, CTV has been diagnosed exploiting direct antibody coated indirect-ELISA (DAC-ELISA) using specific antisera and reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers for the purpose of identification of disease-free mother stock for further development of healthy planting material for the people of this hill regions.

Surveys were conducted in mandarin orchards at 30 locations covering all the blocks/ Taluks in the Darjeeling hills during April to July, 2011. About 3-11 samples (twigs of the tree), from each location were randomly collected and brought to laboratory for diagnostic assay. Virus crude extracts were prepared in extraction buffer at ratio of 1:10 (w/v) macerating 500 mg of tender bark tissues of citrus samples accordingly the method described earlier (Clark and Bar-Joseph, 10). DAC-ELISA using CTV specific antisera and measuring serological colour reaction was carried out using the protocol used earlier (Biswas, 4). Samples of

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healthy and CTV infected mandarin trees maintained in insect-proof greenhouse were used as negative and positive control, respectively. The samples were considered to be positive if the absorbance value in ELISA reader was at least two times more than that of the value of negative control. For RT-PCR, isolation of total plant RNA and synthesis of cDNA was carried out using the protocol used earlier (Biswas, 4, 6). The PCR was performed using the method described by Biswas (6) using specific primer pair KLM 543 and KLM 544 for amplification of complete CP gene of CTV genome.

The biological reactions of Indian CTV isolates in Darjeeling hills were recorded and per cent plant infection was estimated (Table 1). The highest disease incidence was observed in Kalimpong taluk where the mandarin orchards were found to be mostly infected by CTV showing symptoms including prominent chlorosis, poor growth and stunting of plant. A comparatively lesser disease infection was observed in orchards of Darjeeling taluk during the period of survey. The prevalence of aphid vector, *Toxoptera citricidus* was observed in many mandarin

Table 1. Disease incidence and general symptoms caused by *Citrus tristeza virus* in mandarin orchards in different locations of the Darjeeling hills.

Area			No. tree infected /No. tree tested	OD in ELISA (x fold)	RT-PCR positive	Symptom (s)
Sub-division	Block/Taluk	Location				
Kalimpong	Gorubathan	Upper Gorubathan	3/6	1.12-1.53 (4-5)	Yes	Decline and poor growth
		Damlin	6/10	1.34-2.12 (4-7)		
		Pate Gaon	3/6	1.31-1.89 (4-6)		
	Kalimpong	IARI-RS, Kalimpong	2/5	2.25-2.93 (8-10)	Yes	Decline, chlorosis, stunting, poor growth
		Reily road	7/11	1.15-2.54 (4-8)		
		Icchay Basti	10/10	1.55-2.45 (5-8)		
		Chibbo Basti	9/11	1.11-1.89 (4-6)		
	Algarah	Pedong	4/5	1.55-2.03 (5-7)	Yes	Decline, poor growth, chlorosis, stunting
		Sakyong	3/7	0.98-1.57(3-5)		
		13th mile	4/7	0.85-2.13 (3-7)		
Darjeeling	Takdah	Lower Glenburn	5/5	1.01-1.07 (3)	Yes	Decline and chlorosis
		Glenburn	2/3	1.06-1.21 (3-4)		
		Bazaar Gaon	0/3	0.41-0.48 (<1)		
		Singrintam-1	2/3	1.18-1.49 (4-5)		
		Singrintam-2	3/4	1.21-1.52 (4-5)		
		Takling	4/6	1.59-2.43 (4-5)		
		Soreng	6/6	1.23-2.08 (4-9)		
		Mangowa	3/4	1.53-1.87 (5-6)		
	Bijanbari	Upper Rondok Basti	6/9	1.24-1.62 (4-5)		Decline, chlorosis, stunting
		Lower Rondok Basti	4/7	1.08- 1.31 (3-4)		
		Ging TE	10/10	1.82-2.85 (6- 9)	Yes	
		Lebong	3/7	0.98-1.23 (3-4)		
	Sukhia-pokhari	Upper Sukiapokhri	3/6	1.28-1.39 (4-5)	Yes	Poor growth, chlorosis, stunting
		Magarjung	3/7	0.87-1.66 (3-6)		
Karseong	Mirik	Upper Mirik	6/9	0.80-1.81 (3-6)	Yes	Poor growth, chlorosis, stunting
		Soureni	5/8	0.83-1.56 (3-5)		
	Karseong	Lower Monpu	3/4	1.95-2.90 (6-9)		Poor growth, chlorosis, stunting

Av. OD values of positive, healthy and buffer control are 2.92, 0.32 and 0.28 respectively. OD Value taken at 405 nm; x fold titer values were calculated compared with the OD values of infected with healthy control

orchards, particularly in the areas located at lower altitude. Occurrence of the aphid vector is common in the mandarin orchards, with an exception in a few areas located at higher altitude above 1500 m in the Darjeeling hills (Mukhopadyay *et al*., 12; Biswas, 4). The *T. citricidus* transmits CTV efficiently to citrus species in the Darjeeling hills (Biswas, 4).

In the present study, ELISA results showed that mandarin samples of 29 out of total 30 locations were CTV positive with considerably higher virus titer (OD value). Only samples from Bazaar Gaon were found to be ELISA negative but the orchards in these regions are truly free from CTV infection is yet to be confirmed by further studies and analysis. Infected citrus samples showed different titer values ranging from 3 to 10-fold compared to healthy control in ELISA reader (Table 1). The virus infection detected by ELISA was further confirmed by RT-PCR. Total number of seven ELISA positive citrus samples was randomly tested and all of them were found to be RT-PCR positive amplifying desired size of 672 nucleotide sequence band of CP gene from the CTV genome (Fig.1). The present results showed that per cent tree infection in some orchards were high, for instance, all the test samples from orchards of Lower Glenburn and Soreng in Takda block, Icchay Basty in Kalimpong block and Ging TE in Bijanbari block were found to be CTV positive. The previous (Biswas, 4) and present studies concluded that CTV is widely distributed in all the mandarin growing areas of eight blocks in the Darjeeling hills.

In the present study, it was observed that CTV causes a general decline symptom along with chlorosis, poor growth and stunted growth of the mandarin tree in the majority of the orchards in the Darjeeling hills. Previously, Indian isolate Kpg3 has been described earlier to be a declining inducing CTV isolate in the Darjeeling hills (Biswas *et al*., 7). In the present study, stem pitting symptoms was not observed in any of the orchard in this region surveyed. Therefore, based on symptomatology, the previous (Biswas *et al*., 7) and the present study concluded that CTV isolates infecting mandarin orchards in the Darjeeling hills are decline inducing isolates.

Therefore, use of virus-free planting materials and control of insect vectors are essential that would keep citrus industry more viable and profitable. A strategy for production of CTV free mandarin planting material for the Darjeeling hill have been developed (Biswas *et al*., 5). The present study focused on the use of bud wood certification programme and thereby production and supply of CTV-free planting materials, which are the universally recognized method for replenishing of the declined citrus orchards.

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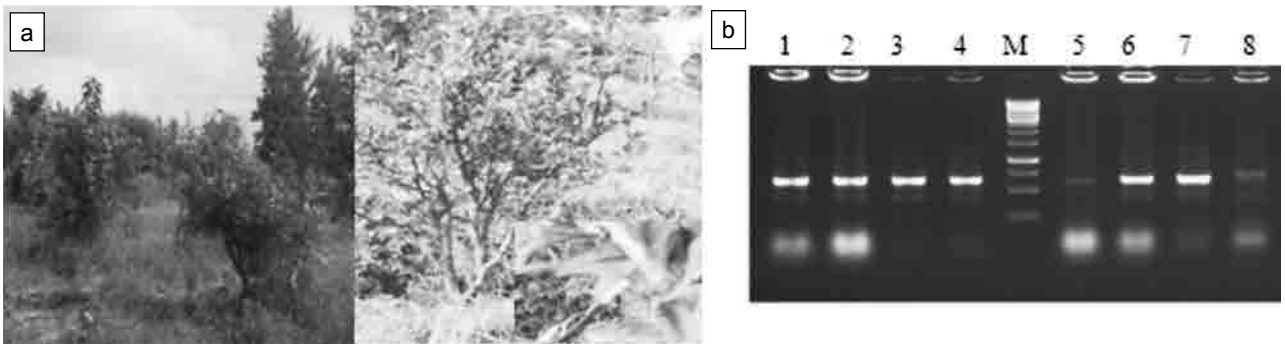

Fig. 1. a. Mandarin orchard showing decline symptoms with chlorosis and poor growth in the Darjeeling hills; b. Gel electrophoresis analysis showing RT-PCR amplification of CP gene of CTV genome of different citrus samples, Lane M: 1 kb DNA ladder, lane 1: CTV infected green house mandarin plant as positive control, lane 2: sample of Gorubathan, 3: IARI-RS, Kalimpong, 4: Pedong, 5: Lower Glenburn, 6: Ging TE, 7: Upper Mirik and 8: Upper Sukhiapokhri.

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