Int.J.Curr.Microbiol.App.Sci (2019) *8*(9): 749-757

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 09 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.809.090

Biological Indexing and Molecular Approaches in Detecting a Mild Strain 'CRS 4' against *Citrus tristeza* Virus in Khasi Mandarin (*Citrus reticulata*)

Borsha Rani Baruah[1*], R. K. Kakoti[2], A. Borbora[2], S. Singh[3], S. Saikia[4] and P. D. Nath[1]

[1]Department of Plant Pathology, [3]Department of Agricultural Biotechnology, Assam, India Agricultural University, Jorhat-13, India
[2]Citrus Research Station, Tinsukia, 4Horticultural Research Station, Kahikuchi, Assam, India Agricultural University, Assam, India

**Corresponding author*

Keywords

Citrus tristeza virus, Khasi mandarin, mild strain, biological indexing, BD/RT-PCR, Assam

Article Info

Accepted:
10 August 2019
Available Online:
10 September 2019

A B S T R A C T

Citrus tristeza virus, the most important viral disease of citrus, is reported in Assam and other NE states of India to infect Khasi mandarin (*Citrus reticulata*), the most economically important citrus crop of the region. For effective management, an attempt was made to identify a potential mild isolate against virus. Leaf samples were collected from Khasi Mandarin plants expressing differential symptoms from three different locations *viz*., Tinsukia, Golaghat and Mariani of Upper Brahmaputra Valley Zone of Assam. These were then grouped into three categories based on ELISA OD_{405} values. Biological indexing with CTV positive samples from these three serological categories on Mexican lime (*Citrus aurantifolia*) seedlings resulted in symptom expression within three months post grafting. Visible symptoms of CTV infection were observed in some of the graft successful indicator plants whereas, in Khasi mandarin selection 'CRS 4', no visible symptom development took place within this period. Based on the results, the plants were grouped into two groups- symptom producing and non-symptom producing, and were confirmed through Bi-directional RT-PCR with mild and severe strain primers. PCR products for 'CRS 4' were sequenced. Consensus sequences showed a single nucleotide difference at position 371 for mild isolates (CRS 4), thereby confirming the identity.

Introduction

Citrus is one of the most lucrative fruit crops in India possessing an enduring potential in the international trade, cultivated in an area of 1,003 thousand ha with a production of 12,546 thousand MT (Anon., 2018). Northeast India is one of the 25 globally acclaimed biodiversity hotspots and known as one of the centers of origin of diverse citrus species. Amid all the citrus crops cultivated in the region, Khasi mandarin (*Citrus reticulata*) is the one of highest commercial value and maximum area under its cultivation is found in Assam and Meghalaya (Singh *et al.*, 2016).

Citrus dieback or citrus decline is a dateless hurdle of citrus cultivation in India and *Citrus tristeza virus* (CTV) being the major blameworthy (Ahlawat, 1997). CTV is the most important viral disease of citrus (Bar-Joseph *et al.*, 1989) as since its first outbreaks in South America in the 1940s it was reported to be the cause of death of more than 100 million trees worldwide (Bar-Joseph *et al.*, 2010). Hence it was very appropriately quoted by Moreno *et al.*, (2008) that "CTV is a pathogen that changed the course of citrus industry". In India, CTV infection has been reported in most of the commercial species of citrus (Ahlawat, 1997; Biswas, 2008; Kishore *et al.*, 2010; Borah, 2011; Kashyap *et al.*, 2013). The first occurrence of the disease was reported in Assam by Bhagabati *et al.*, (1989) and since then various reports of its presence were found (Borah, 2011; Kashyap *et al.*, 2013; Singh *et al.*, 2017).

Virus infections were quite difficult to control, quarantine restrictions and bud-wood certification being the main approaches for its management. But in locations where the disease and its effective aphid vectors were endemic, cross-protection by the purposive introduction of mild strains of the virus into virus-free propagating material being the only resort (van Vuuren and Breytenbach, 2011). Cross-protection has constantly proved to play a vital role in sustaining profitability of citrus production around the globe (Moreno *et al.*, 2008), being a means to extend the economic life of the crop (Lee *et al.*, 1992). However for effective accomplishment of the cross-protection technique, detection and accurate selection of potential mild isolates being factor of prime importance. Traditional biological indexing in indicator host (Roistacher, 1991) along with modern molecular techniques like bi-directional PCR (Cevik *et al.*, 1996; Roy and Ramachandran, 2002) or sequencing (Pappu *et al.*, 1993) provides a base for the strain differentiation and identification.

As northeastern part of India being the natural home of citrus with a number of citrus species/varieties originating from this region (Sharma *et al.*, 2004; Singh *et al.*, 2016), so there was a great probability of detecting mild strains of the virus in this region. It has also been found that CTV mild isolates that were selected in the same region where they were used were superior to isolates obtained from other areas (Zanutto *et al.*, 2013). Moreover, Khasi mandarin (*Citrus reticulata*) selection 'CRS 4' maintained in Citrus research station, Tinsukia were reported to be healthier looking and bearing a good crop in comparison to the other Khasi mandarin plants (unpublished report). Therefore, the present investigation was carried out to identify a potential mild isolate of Khasi mandarin in an attempt to combat CTV.

Materials and methods

Sample collection and serological grouping

For CTV strain differentiation, leaf samples from hundred Khasi Mandarin (*Citrus reticulata*) plants expressing differential symptoms were collected from Tinsukia, Golaghat and Mariani of Upper Brahmaputra Valley Zone of Assam. These were then

assayed by commercial DAS-ELISA Kit (Bioreba, AG, Switzerland) as per recommended protocol and grouped into three categories *viz.* low range, medium range and high range based on ELISA OD_{405} values.

Biological indexing

Twenty plants from each category were selected for biological indexing. Two to three leaf-pieces from these plants were then inoculated to one year old Mexican lime or *Kaghzi* lime (*Citrus aurantifolia*) seedlings following the procedure of Roistacher (1991) (Fig. 1 A). The seedlings were maintained under insect free condition in net house till three months for symptom expression (Fig. 1 B). Based on the symptom development, plants were grouped as symptom producing and non-symptom producing, and leaf samples from these plants, six months post inoculation, were subjected to reverse transcriptase polymerase chain reaction (RT-PCR).

Extraction of total RNA

Total RNA extraction was carried out by a standardized laboratory protocol using Triazole. About 100 mg of leaf tissue was homogenized under liquid nitrogen and 1 mL of Triazole was added to it. 200 µL Chloroform added to the solution, incubated in ice for 15 min followed by centrifugation for 15 min at 12,000 rpm. Aqueous phase transferred to a new tube and 0.5 mL isopropanol was added, followed by incubation in ice for 10 min. The solution was centrifuged for 10 min at 12,000 rpm and on removing the supernatant, the RNA pellet was washed with 1 mL 70 % ethanol by centrifuging at 7500 rpm for 10 min. The RNA pellet was dissolved in 40 µL of RNase free water and stored at -45^{0}C. Quantity and purity of the extracted RNA from the samples were measured in the Bio-Spectrophotometer (Eppendorf) and yielded an average RNA concentration of 744-1050.50 ng/µl with A260/A280 ratio in the range of 1.87-2.01.

Bi-directional reverse transcription-polymerase chain reaction (BD/RT-PCR)

Two internal (CN 218 and CN 219) and two terminal (CN 119 and CN 120) primers (Cevik *et al.*, 1996) yielding band sizes of 672 bp for whole CP gene and 400 bp and 300 bp for mild isolates and severe isolates respectively were used for the detection. For each RT-PCR, a 10 µL reaction mixture was prepared using Takara PrimeScript^{TM} One Step RT-PCR Kit Ver. 2 containing 0.4 µL of Prime Script 1 step Enzyme Mix, 5.0 µL of 2 X 1 step buffer, 0.4 µL of each forward primer and reverse primer, 3.0 µL of RNase free H_20 and 0.8 µL of RNA template. PCR was run in a thermal cycler (Agilent Technologies) with PCR conditions: reverse transcription at 50^0 for 30 min followed by denaturation at 94^{0}C for 1 min, annealing at 50^{0}C for 2 min, elongation at 72^{0}C for 1 min for 40 cycles and final elongation at 72^{0}C for 10 min. 10 µL PCR products mixed with one µL of 6X gel loading dye along with five µL of 100 bp DNA ladder were analyzed in 1.5 per cent agarose gel electrophoresis in 1X TBE containing 0.5 µg/mL of Ethidium bromide. The electrophoretic gel was then run at 50 mAmp till the dye has migrated one-third of the distance in the gel, visualized using a UV transilluminator and the gel images were captured in Gel Doc (Bio-Rad).

CTV strain reconfirmation through sequencing

Strain identification and confirmation through RT-PCR was further reconfirmed through sequencing the mild isolates. RT-PCR products were sent to Bioserve Biotechnologies India Pvt. Ltd, Hyderabad for sequencing in duplicate.

On receiving the sequencing results, the sequences were assembled and consensus sequences were prepared using the Codon Code Aligner software. These were then aligned with the NE isolate (JRT5) (GenBank: KC986383.1) (Kashyap *et al.*, 2013) using the MultAlin software (Corpet, 1988).

Results and Discussion

Serological grouping of CTV positive plant samples

Considering five times the average reading of ELISA OD_{405} values for negative control (0.19) and the two blanks (0.072), the cut off value (0.56) was fixed and three groups were then formed as low range (0.56-1.0), medium range (1.1-1.8) and high range (>1.8) for covering samples from all the virus titre ranges.

Among all the serological methods, enzyme-linked immunosorbent assay (ELISA) is the most popular due to their dependability, rapidity and low relative cost have been widely used for CTV detection across the globe (Nikolaeva *et al.*, 1998; Cambra *et al.*, 2000; Korkmaz *et al.*, 2008; Kishore *et al.*, 2010; Tarafdar *et al.*, 2012). Detection of CTV in Khasi mandarin by ELISA has been reported earlier from Assam and other North-Eastern states of India (Borah, 2011; Kashyap *et al.*, 2013; Singh *et al.*, 2017) with OD_{405} values in the range of 0.689-2.270.

A similar range in OD_{405} values through ELISA were obtained in the present investigation which was in conformity with the earlier results.

CTV strain identification through biological Indexing

Biological indexing with CTV positive samples of the three serological categories, symptom expression was observed on grafted Mexican lime or *Kaghzi* lime (*Citrus aurantifolia*) seedlings within three months post grafting. Visible symptoms of CTV infection like vein clearing, vein darkening and yellowing of the leaves were observed in some of the graft successful indicator plants whereas, in plants grafted with 'CRS 4', no visible symptom development took place within this period. Thereby, the plants were grouped into two groups based on symptom development as symptom producing and non-symptom producing (mild isolates).

Even with the advancement of modern techniques, seedling indexing to Mexican lime still remains a useful tool for detection of CTV and its isolates. Mexican lime or key lime (*Citrus aurantifolia*), *kaghzi* in India, is highly sensitive to tristeza and is the preferred indicator.

Inoculation with two inoculum "buds" (buds with eyes, blind buds or chip buds) or leaf pieces, or a minimum of five or six leaf discs per indicator plant results in symptoms development in over 90 percent of seedlings within nine weeks (Roistacher, 1991).

Vein-clearing symptoms in leaves of Mexican limes could be readily identified in plants inoculated with most CTV isolates.

However, vein-clearing symptoms induced by some mild-reacting isolates may be difficult to perceive, since only a few leaves may exhibit an occasional mild fleck in the vein (Balaraman and Ramakrishnan, 1980; Garnsey *et al.*, 1987; Roistacher, 1991). The results of the present investigation were in queue with the earlier findings.

CTV strain differentiation and confirmation through BD/RT-PCR

Grouping of the plants on the basis of symptom development was confirmed

through one step RT-PCR of the grafted plants with mild and severe strain primers. PCR results depicted that in case mild isolates (CRS 4), there was formation of full coat protein gene (672 bp) and 400 bp DNA fragment whereas, it was 672 bp and 300 bp DNA fragment in the severe isolates (Fig 2 A and B).

Fig.1(A) Leaf-piece grafting in Mexican lime seedlings and (B) Maintenance of the seedlings under net house condition

Fig.2 Agarose gel electrophoresis showing strain differentiation by BD/RT-PCR. (A) Lane M: 100 bp ladder; lane 1: whole CP gene (672 bp) using CN 119/120; lane 2: mild isolate 'CRS 4' (400 bp) using CN 119/219; lane 3: negative control; lanes 4-6: severe isolates (300 bp) using CN 120/218. (B) Lane M: 100 bp ladder; lane 1: negative control; lane 2: mild isolate 'CRS 4' (400 bp and 672 bp) using CN 119/219/218/120; lane 3: severe isolates (300 and 672 bp) using CN 119/219/218/120.

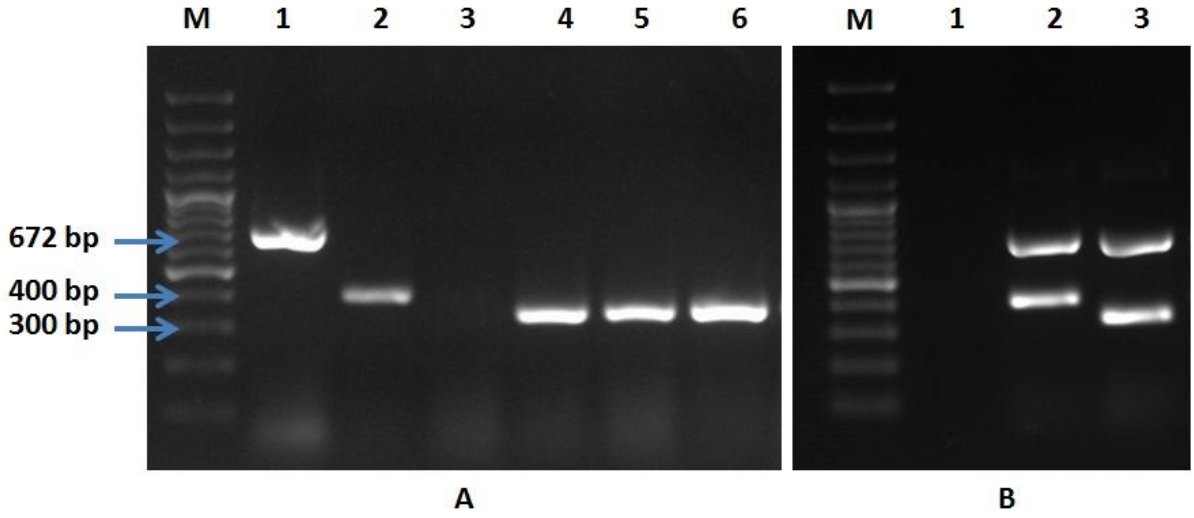
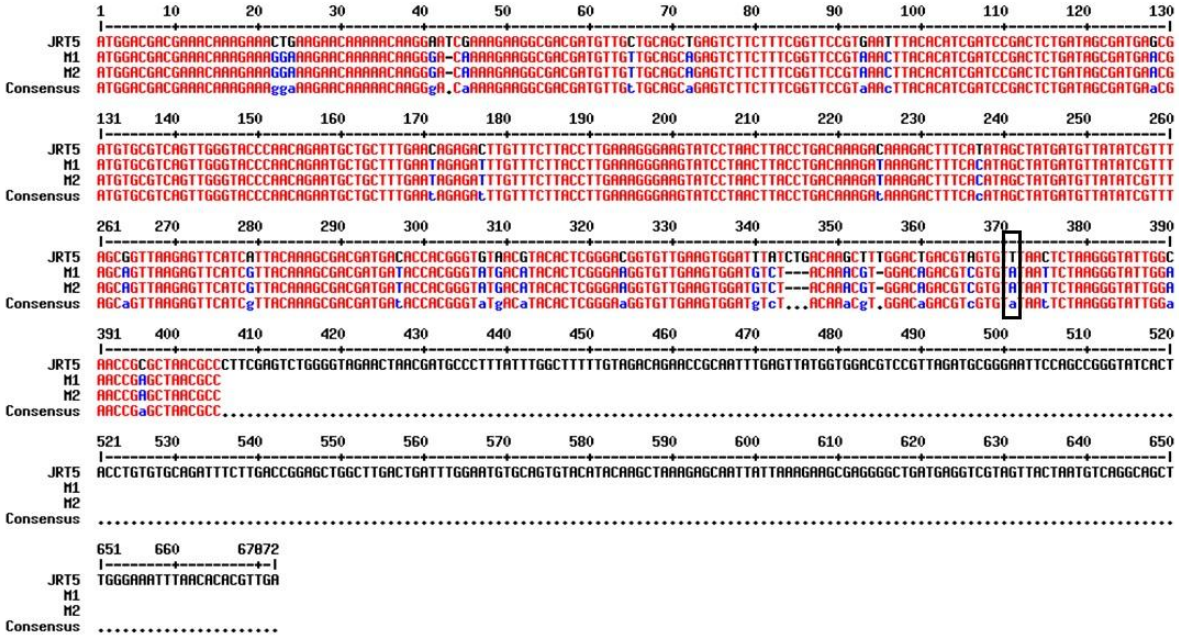


Fig.3 Sequence alignment of CTV JRT5 with mild isolates (CRS 4), nucleotide "A" at position 371 in mild isolates



The development of bi-directional PCR (BD/PCR) for CTV strain differentiation was facilitated by the revelation that MCA-13 epitope was dominated by a single nucleotide (A/T).

Cevik *et al.*, (1996) designed two internal primers, one specific for generally mild (MCA-l3 non-reactive) strains *i.e.* CN 218 and the other specific for generally severe (MCA-13 reactive) strains *i.e.* CN 219, of CTV, with two terminal primers for the ends of CP, *i.e.* CN 119 and CN 120 for strain identification of CTV. In India, Roy and Ramachandran (2002) used this RT-PCR technique for differentiating the strains of CTV on the basis of the amplified product size.

The results obtained in the current investigation were similar to those reported earlier and bi-directional PCR technique was successful in our study in CTV strain differentiation.

CTV strain reconfirmation through sequencing

Sequencing results depicted 400 bp sequences for the 'CRS 4' isolates and on alignment with the NE isolate JRT5, nucleotide "A" was observed at position "371" of the coat protein gene (Fig. 3). Hence confirming the identity of the CTV isolates. The recent advances achieved in the detection techniques during the last few years revealed the underlying differences in the mild and severe strains of CTV.

The development of monoclonal antibody MCA13 was a major breakthrough (Permar *et al.*, 1990), as it reacts only the severe strains but not with the mild ones. A leap in the study of CTV genetics was achieved with the advancements in the sequencing technology (Karasev *et al.*, 1995). Analyzing the capsid coat protein amino acid sequences of various isolates, a constant difference in the amino acid at position "124" was identified. The

amino acid was tyrosine in case of mild strains and phenylalanine in case of severe strains. A single nucleotide (A/T), at position "371", dictates the reactivity of MCA-13 *i.e.* TTT and TAT for MCA-13-reactive and non-reactive strains, respectively (Pappu *et al.,* 1993). The results obtained in the present investigation regarding a single nucleotide (A/T) difference at position "371" in case of mild isolates was in accordance with the previous records.

Citrus tristeza virus (CTV), one of the major contributors of citrus decline, has been recorded to infect different commercial species of Citrus in Assam and other NE states of India (Bhagabati *et al.,* 1989; Kishore *et al.,* 2010; Borah, 2011; Kashyap *et al.,* 2013; Singh *et al.,* 2017). CTV being a major factor for dwindling yield of the crop and therefore calls for an effective management of the virus to substantiate the production at an economic level. The only solution to the current situation is the use of cross-protection technique and for that there is requirement of a potential mild isolate. However, there is no any previous report of mild isolates in this region of India. Therefore, in the present investigation an effort was made to detect a mild isolate of CTV in order to combat the virus infection. The results obtained in the present investigation reports the identification of mild isolate 'CRS 4' in Khasi mandarin against CTV. But there is requirement of some additional long duration studies to evaluate the protective level of the isolate in cross-protection.

References

Ahlawat, Y.S. 1997. Viruses, greening bacterium and viroids associated with citrus (*Citrus species*) decline in India. *Indian J. Agr. Sci.* 67: 51-57.

Anonymous. 2018. Horticultural Statistics at a Glance 2018. Horticulture Statistics Division, Department of Agriculture, Cooperation & Farmers' Welfare, Ministry of Agriculture & Farmers' Welfare, Government of India.

Balaraman, K. and Ramakrishnan, R. 1980. Strain variation and cross protection in *Citrus tristeza virus* on acid lime. In: *Proc. Eight IOCV Con*. pp. 60-68.

Bar-Joseph, M., Batuman, O. and Roistacher, C. 2010. The history of *Citrus tristeza virus*—Revisited. In: *Citrus tristeza virus complex and tristeza diseases*. Karasev, A.V. and Hilf, M.E. (Eds.). APS Press, St Paul, MN, USA. pp. 3-26.

Bar-Joseph, M., Marcus, R. and Lee, R.F. 1989. The continuous challenge of *Citrus tristeza virus* control. *Annu. Rev. Phytopathol*. 27: 291–316.

Bhagabati, K.N., Ahlawat, Y.S., Chakroborty, N.K. and Borthakur, B.C. 1989. Distribution of greening, tristeza and mosaic disease of citrus in North eastern states of India. *Indian Phytopath*. 42: 552-555.

Biswas, K.K. 2008. Molecular diagnosis of *Citrus tristeza virus* in mandarin (*Citrus reticulata*) orchards of Darjeeling hills of West Bengal. *Indian J. Virol.* 19(1): 26-31.

Borah, M. 2011. Identification of *Citrus tristeza virus* on different citrus species. M. Sc. (Agri.) thesis, Assam Agricultural University, Jorhat.

Cambra, M., Gorris, M.T., Marroquina, C., Roman, M.P., Olmos, A., Carmen Martinez, *et al.,* 2000. Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain. *Virus Res*. 71: 85-95.

Cevik, B., Pappu, S. S., Pappu, H. R., Benscher, D., Irey, M., Lee, R. F. and Niblett, C. L. 1996. Application of Bi-Directional PCR to *Citrus Tristeza Virus*: Detection and Strain

Differentiation. In: *Proc. Thirteenth IOCV Con*. pp. 17-24.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res*. 16 (22), 10881-10890.

Garnsey, S.M., Gumpf, D.J., Roistacher, C.N., Civerolo, E.L., Lee, R.F., Yokomi, R. K. and Bar-Joseph, M. 1987. Toward a standardized evaluation of the biological properties of *Citrus tristeza virus*. *Phytophylactica*. 19: 151-157.

Karasev, A., Boyoko, V., Gowda, V., Nikolaeva, O.V., Hilf, M.E., Koonin, M., *et al.*, Complete sequence of the *Citrus tristeza virus* RNA genome. *Virology*. 208: 511−520.

Kashyap, A., Acharjee, S. and Nath, P.D. 2013. Serological and Molecular Detection of in Citrus Fruit Species of North Eastern Region of India. *J. Mycol. Plant Pathol.* 43(4): 431-435.

Kishore, K., Rahman, H., Kalita, H., Pandey, B. and Monika, N. 2010. Prevalence of *Citrus tristeza virus* in Mandarin of Sikkim Himalayan Region. *Indian. J. Virol*. 21(2):140-143.

Korkmaz, S., Cevik, B., Onder, S., Koc, K. and Bozan, O. 2008. Detection of *Citrus tristeza virus* (CTV) from Satsuma Owari mandarins (*Citris unshiu*) by direct tissue blot immunoassay (DTBIA), DAS- ELISA, and biological indexing. *New Zeal. J. Crop Hort*. 36(4): 239-246.

Lee, R.F., Niblett, C.L. and Derrick, K.S. 1992. Mild strain cross protection against severe strains of *Citrus tristeza virus* in Florida. In: *Proc. First Int. Sem. Citriculture, Pak*. pp. 400-405.

Moreno, P., Ambros, S., Albiach-Marti, M.R., Guerri, J. and Pena, L. 2008. *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. *Mol. Plant Pathol*. 9(2): 251–268.

Nikolaeva, O.V., Karasev, A.V., Garnsey, S.M., and Lee, R.F. 1998. Serological differentiation of the citrus tristeza virus isolates causing stem pitting in sweet orange. *Plant Dis*. 82:1276-1280.

Pappu, H. R., Pappu, S. S., Manjunath, K. L., Lee, R. F. and Niblett, C. L. 1993. Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. *Proc. Natl. Acad. Sci. USA*. 90: 3641-3644.

Permar, T. A., Garnsey, S. M., Gumpf, D. J. and Lee, R. F. 1990. A monoclonal antibody that discriminate strains of citrus tristeza virus. *Phytopathology*. 80: 224-228.

Roistacher, C.N. 1991. A handbook for detection and diagnosis of graft-transmissible diseases of citrus. Food and Agriculture Organization of the United Nations. pp. 17-34.

Roy, A. and Ramachandran, P. 2002. Bi-directional PCR- a tool for identifying strains of *Citrus tristeza virus*. *Indian Phytopath*. 55(2):182-186.

Sharma, B.D., Hore, D.K. and Gupta, S.G. 2004. Genetic Resources of Citrus of north-eastern India and their potential use. *Gen. Resour. Crop Evol*. 51:414-418.

Singh, A.K., Meetei, N.T., Singh, B.K. and Mandal, N. 2017. High incidence of citrus tristeza virus in mandarin (*Citrus reticulata*) in North-East states of India. *Virus Dis*. 28(4): 401-407.

Singh, A.M., Meetei, N.T., Singh, B.K. and Mandal, N. 2016. Khasi mandarin: its importance, problems and prospects of cultivation in North-eastern Himalayan region. *Int. J. Agric. Environ. Biotechnol*. 9(4): 573-592.

Tarafdar, A., Ghosh, P.D. and Biswas, K.K. 2012. In planta distribution, accumulation, movement and

persistence of *Citrus tristeza virus* in citrus host. *Indian Phytopath.* 65(2): 184-188.

Van Vuuren, S.P. and Breytenbach, J.H.J. 2011. Transmission and movement of potential *Citrus tristeza* Virus cross-protection sources in four soft citrus cultivars under greenhouse conditions. *S. Afr. J. Plant & Soil*. 28(1): 43-48.

Zanutto, C.A., Corazza, M.J., de Carvalho Nunes, W.M. and Muller, G.W. 2013. Evaluation of the protective capacity of new mild *Citrus tristeza virus* (CTV) isolates selected for a pre-immunization program. *Sci. Agric.* 70(2): 116-124.

How to cite this article:

Borsha Rani Baruah, R. K. Kakoti, A. Borbora, S. Singh, S. Saikia and Nath, P. D. 2019. Biological Indexing and Molecular Approaches in Detecting a Mild Strain 'CRS 4' against Citrus tristeza Virus in Khasi Mandarin (*Citrus reticulata*) *Int.J.Curr.Microbiol.App.Sci.* 8(09): 749-757. doi: https://doi.org/10.20546/ijcmas.2019.809.090