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# Detection of Mandarivirus and greening bacterium using electron microscopy, PCR and RT-PCR in Kinnow mandarin nurseries in Punjab

R P PANT[1], RAKESH KUMAR[2], ANITA ARORA[3] and V K BARANWAL[4]

*ICAR-Indian Agricultural Research Institute, New Delhi 110 012*
*and Punjab Agricultural University, Ludhiana 141 004*

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ABSTRACT

Kinnow mandarin is one of the most important fruit crops in northern India particularly in Punjab. Indian citrus ringspot virus (ICRSV), Citrus yellow vein clearing virus (CYVCV) and citrus greening bacterium (CGB) are very important graft transmissible pathogens causing reduced productivity in Kinnow mandarin. A survey was undertaken in 11 kinnow mandarin nurseries, 2 to 4 star rated by National Horticultural Board in Punjab. Samples collected from these nurseries were indexed by electron microscopy, immunosorbent electron microscopy (ISEM), PCR and RT-PCR. In RT-PCR test, out of 33 samples of kinnow mandarin mother plants, 3 samples were found positive for CYVCV and 6 samples for ICRSV. In two nurseries, both the viruses were detected in the same plant indicating mixed infection in the mother plants. In ISEM test, 4 samples out of 12 samples were positive for ICRSV and 2 samples were positive for CYVCV. CGB was detected in two plants out of nine plant leaf samples collected from four nurseries. In view of these studies it is evident that molecular diagnostics as indexing tool need to be applied for the production of clean planting material of kinnow mandarin.

**Key words:** Citrus greening bacterium, Indexing, ISEM, Kinnow mandarin, Mandarivirus, PCR, RT-PCR

Kinnow mandarin is one of the most important fruit crops in Punjab, Rajasthan, Haryana, Delhi, Himachal Pradesh, Jammu and Kashmir and parts of Uttar Pradesh. It is known for its production, productivity, juice content and fruit quality. Kinnow mandarin is a hybrid of King and Willow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenoa) and introduced in India in 1954 from University of California, Riverside by J C Bakshi at the Punjab Agricultural University, Regional Fruit Research Station, Abohar. The interest of farmers in adoption of Kinnow cultivation is increasing day by day due to suitable agro-climatic conditions, higher crop yield and demand in domestic and international market. Over the years, the crop has been affected by several graft transmissible pathogens such as CTV of Closterovirus, ICRSV of *Mandarivirus*, CGB, fastidious bacterium. C*itrus yellow vein clearing virus* (CYVCV) is another *Mandarivirus* which was reported recently in etrog citron (Alshami *et al*. 2003). Ringspot disease of citrus was first described by Ahlawat (1989) on Kinnow mandarin mother plant at Punjab Agricultural University, Abohar. The incidence of ICRSV had been reported up to 100% on Kinnow mandarin orchards in Punjab (Ahlawat and Pant 2003). CYVCV was first reported from Abohar, Punjab in 2003. Both the viruses have been characterized and their viral genomes have been fully sequenced and placed as distinct species in the genus *Mandarivirus*, under the family *Alphaflexiviridae* (Rustici *et al*. 2002, Adams *et al*. 2004, Adams *et al*. 2014).

As large numbers of nurseries are producing kinnow mandarin saplings, a study was undertaken to ascertain the infection of Mandarivirus and citrus greening bacterium (CGB) in selected nurseries of Punjab. These nurseries are rated by National Horticultural Board (NHB), Ministry of Agriculture and Cooperatives and Farmers Affairs, Government of India. The assessment of nurseries is based on evaluation of parent material, propagation in disease free condition by adoption of technically prescribed method, adoption of good nursery management practices, reliable record keeping and training of staff. After examining each parameter, the assessment team provides 0-5 star rating to the nurseries. There are about 32 citrus nurseries identified by NHB in different locations in Punjab. Survey was conducted in 11 nurseries at Ludhiana, Fazilka, Hoshiyarpur and Abohar in collaboration with Department of Fruit and Vegetable Crops, PAU, Ludhiana.

[1]Principal Scientist (e mail: rajendrappant@gmail.com), [2]Senior Research Fellow (e mail: rakesh.iari07@gmail.com), [4]Professor and Incharge (e mail: vbaranwal2001@yahoo.com), Plant Virology Unit, Division of Plant Pathology, IARI, New Delhi, [3]Assistant Plant Pathologist (e mail: anitapau@pau.edu), Department of Fruit Science, College of Agriculture, Punjab Agricultural University, Ludhiana.

## MATERIALS AND METHODS

The information about star rated nurseries of kinnow mandarin is available on the website of NHB. 11 star rated nurseries (2-4 star) were surveyed in Ludhiana, Fazilka, Hoshiyarpur and Abohar. Leaves showing ringspot, yellowing, vein clearing, oak leaf pattern and mottling symptoms as well as leaves from healthy non symptomatic plants were collected and processed for electron microscopy, ELISA, PCR and RT-PCR. Samples were graft inoculated on healthy Kinnow plants and maintained in glass house.

Crude extracts from leaf tissues of 5 healthy and 12 symptomatic infected plants of Kinnow mandarin were homogenized in 0.07 M phosphate buffer, pH 6.5, negatively stained in 2% uranyl acetate and observed under JEOL-1011, transmission electron microscope (TEM). Digital images were recorded by Olympus CCD camera MEGAVIEW G2 Soft Imaging System attached to the EM interface. Immunoelectron microscopy (IEM) was performed as described by Milne (1993) using 1:50 dilutions of ICRSV and CYVCV antiserum.

RT-PCR of total 33 samples collected during the survey was performed for ICRSV and CYVCV. Total RNA was extracted from 100 mg of symptomatic and healthy leaf samples of kinnow mandarin using the RNeasy Plant Mini Kit. Positive samples of ICRSV and CYVCV maintained in green house also used for RNA isolation. Two step Reverse transcription-polymerase chain reaction (RT-PCR) was performed. In first step, cDNA preparation was carried out by using Verso cDNA synthesis Kit. A reverse transcription reaction mixture 20 µl was prepared which consisted of 1.5 µl of total RNA, 4 µl of 5 × cDNA synthesis buffers, 2 µl of dNTPs, 1 µl of RNA primer, 1 µl of RT enhancer, 1 µl of Verso Enzyme Mix and 9.5 µl of $ddH_2O$. Reverse transcription conditions were 42°C for 30 min and 95°C for 1 min.

After cDNA preparation, PCR was performed using virus specific primers designed from partial RdRp and CP sequences for ICRSV and partial CP for CYVCV as described previously (Meena and Baranwal 2016). The forward and reverse primer used in the study for ICRSV were 5'AGCGTAACCATTCTTACGCCC3' and 5'GGTTCAGGAGCCTTCGTCTG3' and for CYVCV 5'CTCTCTACCGCAGCTATCCA3' and 5'GGTTAAAGGCAGGTGGAGAG3'. A PCR reaction mixture of 25 µl was prepared which consisted of 1 µl of cDNA, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP (10 mM), 0.5 µl Forward primer (10 pmol/µl), 0.5 µl Reverse primer (10 pmol/µl), 2.5 µl Standard Taq Buffer (10×), Taq DNA Polymerase, 0.5 µl (1.0 unit) and 18 µl Nuclease free water. The amplification conditions were as follows: 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min, and a final 72°C for 10 min. The amplified products were visualised on 1% agarose gel and viewed under gel documentation system.

A total of 9 samples collected from four nurseries were subjected for PCR amplification for detection of citrus greening bacterium. DNA was extracted from 100 mg of healthy and infected leaf midribs using DNeasy Plant mini kit according to manufacturer's directions. Positive control of CGB maintained in the glass house. The forward and reverse primer pair was taken from 16S Ribosomal RNAs. Primer sequences: 5'TGGGTGGTTTACCATTCAGTG3' and 5'CGCGACTTCGCAACCCATTG3' as described previously by Meena and Baranwal (2016) were used. A PCR reaction mixture 25 µl was prepared which consisted of 3 µl of DNA, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP (10 mM), 0.5 µl Forward primer (10 pmol/µl), 0.5 µl Reverse primer (10 pmol/µl), 2.5 µl Standard Taq Buffer (10X) , 0.5 µl Taq DNA Polymerase and 16 µl Nuclease free water. The amplification conditions were as follows: 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 50 s, and a final 72°C for 10 min. The amplified products were checked on 1% Agarose gel and viewed under gel documentation system.

## RESULTS AND DISCUSSION

Ringspot and yellow vein clearing diseases are two important viral diseases affecting commercial cultivation of kinnow mandarin in this region. ICRSV and CYVCV are two virus species under genus Mandarivirus which are associated with these symptomatic citrus plants have been characterized biologically as well as at molecular level (Ahlawat 1989, Pant *et al*. 1999, Ahlawat and Pant 2003, Alshami *et al*. 2003, Rustici *et al*. 2000 and 2002). In the survey of 11 nurseries at different locations of Punjab, symptoms of oak leaf pattern, yellow ringspot and vein clearing were observed in one mother plant of Kinnow mandarin in mother block section at Fruit Plant Nursery, Department of Horticulture, PAU Ludhiana rated 4 stars by NHB (Fig 1). Typical ringspot symptoms were observed on leaves on three kinnow mother plants in Guru Nanak Fruit nursery, Sweta fruit nursery at Fazilka (3 star) and Farmers First Nursery at Maujgarh, Abohar (2 star) (Fig 2). Yellowing of veins and blotchy mottling symptoms on leaves were observed in 4 plants at Hoshiyarpur, Premjit Nursery and Manjit Fruit Nursery at Bunga (both 3 star) (Fig 3).

Fig 1 Kinnow leaves showing oak leaf pattern and ringspot symptoms

Fig 2 Kinnow leaves showing ringspot symptoms

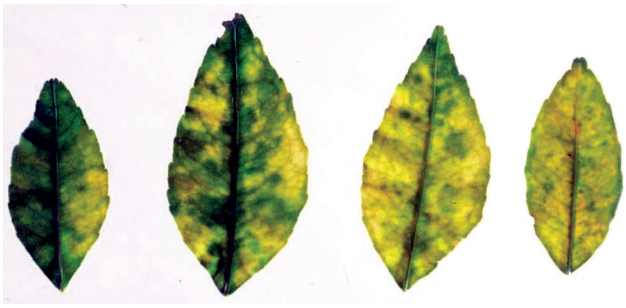

Fig 3 Kinnow leaves showing mottling and yellowing symptoms

Confirmation of viruses and citrus greening bacterium associated with these symptomatic plants, were examined under EM and were further subjected to PCR/RT-PCR. The indexing results of 11 nurseries against Mandarivirus and greening bacterium are summarized in Table 1.

*Electron microscopy*

Initially, the filamentous viruses were detected by electron microscopy but specific detection of ICRSV or CYVCV could be achieved by ISEM using polyclonal antibodies available at Advanced Centre for Plant Virology (ACPV), IARI. Flexuous filamentous particles measuring 685 × 14 nm were observed with oak leaf pattern and ringspot symptoms in 2 samples out of 12 samples. In immunosorbent electron microscopy, these particles could be decorated by CYVCV antiserum test thus confirming the presence of CYVCV. Four samples showing ringspot symptoms showed flexuous filamentous particles measuring 650 × 13 nm and they could be decorated with ICRSV antiserum which confirmed the presence of ICRSV. Over all in ISEM test, out of 12 samples, 2 samples were found positive to CYVCV, 4 samples were found positive for ICRSV out of 10 samples tested. In none of the sample presence of both the viruses could be found.

*Direct antigen coating – enzyme linked immunosorbent assay (DAC-ELISA)*

The specific detection of both viruses in DAC-ELISA was not successful (data not shown). The commercial ELISA kits are not available for both ICRSV and CYVCV, therefore, nucleic acid based RT-PCR was used for their specific detection.

*Reverse transcription- polymerase chain reaction (RT-PCR)*

Table 1 Indexing results showing presence of *Citrus yellow vein clearing virus* (CYVCV), *Indian citrus ringspot virus* (ICRSV) and citrus greening *(Candidatus Liberibacter asiaticum*) diseases in mother block of Kinnow mandarin plants on star rated nurseries in Punjab

| Name of nursery (Mother block) / location | Total no. of mother plant | Total sample tested | Symptoms | Virus indexing | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | CYVCV | | ICRSV | | CGB |
| | | | | ISEM | RT-PCR | ISEM | RT-PCR | PCR |
| Fruit Plant Nursery, Department of Horticulture, PAU, Ludhiana**** | 95 | 14 | Yellow vein/ringspot (4) | (1/5) | (1/14) | (0/5) | (0/14) | ND |
| Shweta Fruit Nursery, Fazilka*** | 55 | 04 | Ringspot (4) | (0/2) | (0/2) | (1/1) | (2/2) | ND |
| Farmer First Nursery-I, Abohar** | 521 | 06 | Ringspot (6) | (1/1) | (1/3) | (1/1) | 1/3) | ND |
| RRS, Abohar-I*** | 100 | 02 | Non –symptomatic | ND | (0/2) | ND | (0/2) | ND |
| Guru Nanak Fruit Nursery, Fazilka.*** | 160 | 03 | Ringspot (3) | (0/3) | (0/3) | (2/2) | (3/3) | ND |
| Parmjit Nursery, Bhunga, Hoshiarpur*** | 22 | 02 | Mottling (2) | ND | (1/2) | ND | (0/2) | 1/2 |
| Farmers First Nursery-II, Abohar** | 35 | | Non-symptomatic | | | | | |
| RRS, Abohar-II*** | 10 | | Non –symptomatic | | | | | ND |
| Manjit Fruit Nursery, Bhunga, Hoshiarpur *** | 28 | 02 | Mottling (2) | ND | (0/2) | ND | (0/2) | 1/2 |
| Center of excellence for fruits. | 50 | 03 | Non-symptomatic | 0/1 | (0/3) | 0/1 | (0/3) | 0/3 |
| Govt. Garden and Nursery, Hoshiarpur** | 31 | 02 | Mottling (2) | ND | (0/2) | ND | (0/2) | 0/2 |

Total number of samples infected/total number of samples tested: ICRSV (8/33); CYVCV (2/33); Citrus greening (2/9); *star rate given by NHB; ND= not done

RT-PCR results revealed that CYVCV was present in 3 samples one each at Fruit Plant Nursery, Department of Horticulture, PAU, Ludhiana; Guru Nanak Fruit Nursery, Fazilka and Farmers First Nursery, Abohar. Amplification of ICRSV was observed in 6 samples, 3 samples from Guru Nanak Fruit Nursery, Fazilka and 2 samples from Sweta fruit Nursery, Fazilka and one sample from Farmers First Nursery, Abohar. A mixed infection of ICRSV and CYVCV was observed from Guru Nanak Fruit Nursery, Fazilka and Farmers First Nursery, Abohar. The amplicons size of 758 bp and 333 bp were obtained from ICRSV and CYVCV positive samples respectively (Fig 4).

*Polymerase chain reaction (PCR)*

PCR for citrus greening bacterium, *Ca. L. asiaticus*, in nine samples collected, only one sample each from Premjit Nursery and Manjit Nursery, Bunga, Hoshiyarpur showed the amplification of greening specific amplicon of 451 bp. Samples collected from Government Garden and Nursery, Hoshiyarpur and Centre of Excellence of Fruit did not show any amplification for greening bacterium, *Ca. L. asiaticus* (Fig 5). Greening bacterium could be detected in two nurseries in PCR test. This indicated that mother plants are not free from these viruses and greening bacterium and this leads to spread of these pathogens in citrus saplings being used by farmers.

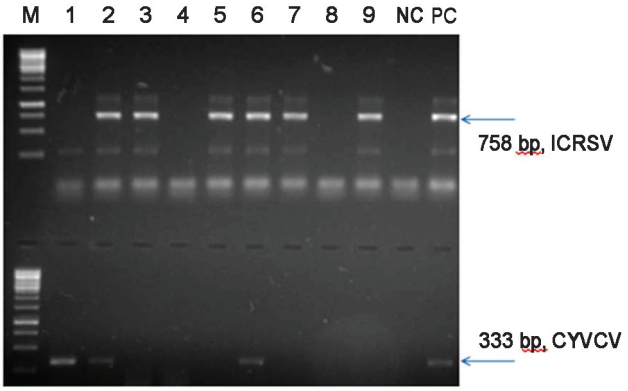


Fig 4 RT-PCR of sample collected from different nurseries. M= marker; Lane 1=Fruit Plant Nursery, PAU, Ludhiana; Lane 2 = Farmers First Nursery, Abohar Lane-3=, Sweta Fruit Nursery, Lane 4= RRS, Abohar and 5, 6, &7= Guru Nanak Fruit Nursery, Fazilka; Lane 8=Paramjit Nursery; Lane 9=Sweta Fruit nursery, Fazilka showing ICRSV and CYVCV. NC= negative control, PC= positive control

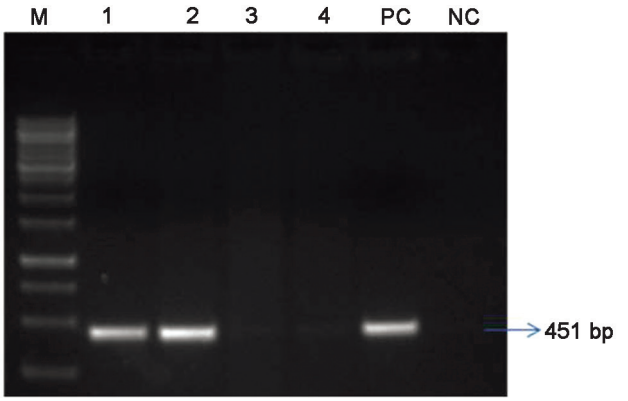


Fig 5 PCR of greening bacterium. M –Gene Ruler 1 kb DNA Ladder, 1-Parmjit Nursery, Bhunga, Hoshiarpur; 2 -Manjit Fruit Nursery, Bhunga, Hoshiarpur; 3 - Center of excellence for fruits; 4 - Govt. Garden and Nursery, Hoshiarpur. PC- Positive control, NC- Negative control

Citrus being a vegetatively propagated crop, infected bud wood plays an important role in spread of these viruses. Commercial citrus growers in the state produce large number of kinnow saplings without proper indexing of mother plants/saplings against viruses and greening bacterium. Indexing of mother plants for known graft transmissible pathogens may not have been done due to lack of proper knowledge on indexing methodology. Contamination of mother plants in different nurseries with Mandarivirus and greening bacterium indicate that there is a need of implementation of virus indexing programme in all the nurseries. Despite very good initiatives taken by NHB to improve the quality and cleanliness of citrus nurseries in the state of Punjab, virus indexing of all mother plants needs to be made mandatory. Stakeholders need to apply molecular diagnostics available against Mandarivirus and greening bacterium for the production virus-free planting material of Kinnow mandarin which will eventually help the farmer in increasing the production and productivity of the crop in this region.

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## REFERENCES

Adams M J, Lefkowitz E J, King A M Q and Carstens E B. 2014. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses. *Archives of Virology* **159:** 2831–41.

Adams M J, Antoniw J F, Bar-Joseph M, Brunt A A, Candresse T, Foster G D, Martelli G P, Milne R G, Zavriev S K and Fauquet C M. 2004. The new plant virus family Flexiviridae and assessment of molecular criteria for species demarcation. *Archives of Virology* **149**: 1045–60.

Ahlawat Y S. 1989. Psorosis-A disease of citrus in India. *Indian Phytopathology* **42**: 21–5.

Ahlawat Y S and Pant R P. 2003. Major virus and virus-like diseases of citrus in India, their diagnosis and management**.** *Annual Review of Plant Pathology* **2:** 447–74**.**

Alshami A A A, Ahlawat Y S and Pant R P. 2003. A hitherto unreported yellow vein clearing disease of citrus in India and its viral etiology. *Indian Phytopathology* **56:** 422–7.

Meena R P and Baranwal V K. 2016. Development of multiplex polymerase chain reaction assay for simultaneous detection of clostero-, badna- and mandari-viruses along with huanglongbing bacterium in citrus trees. *Journal of Virological Methods* **235**: 58–64.

Milne R G. 1993. Electron microscopy of *in vitro* preparations. (*In*) *Diagnosis of Plant Virus Diseases,* pp 215–52. Matthews R E F (Eds.). CRC Press, Baton Rouge.

Pant R P, Baranwal V K, Singh S P and Ahlawat Y S. 1999. Mechanical transmission of Indian citrus ringspot virus (ICRSV). (*In*) Hi-Tech Citrus Management. *Proceedings of International Symposium on Citriculture,* NRCC, Nagpur, pp 1021–3.

Rustici G, Accotto G P, Noris E, Masenga V, Luisoni E and Milne R G. 2000. Indian citrus ringspot virus: a proposed new species with some affinities to potex-, carla-, fovea- and allexiviruses. *Archives of Virology* **145(**9): 1895–908.

Rustici G, Milne R G and Accotto G P. 2002. Nucleotide sequence, genome organisation and phylogenetic analysis of Indian citrus ringspot virus. *Archives of Virology* **147:** 2215–22.