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Full Length Article

Citrus psorosis virus: A Qualitative and Quantitative Detection by RT-PCR and Real-Time Taqman RT-PCR in Pakistan

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Abstract

Psorosis is universally upsetting disease of Citrus triggered by an infectious filamentous ophiovirus named as *Citrus psorosis virus* (CPsV). Reverse transcription–polymerase chain reaction (RT-PCR) and Real-time TaqMan RT-PCR assays were developed for the detection of CPsV in citrus trees from Punjab region of Pakistan. Total RNA was extracted from the sweet oranges and mandarin varieties, which were then converted to cDNA to be used as a template in a conventional PCR using primers for the universal coat protein detection of CPsV. A total of 200 samples were screened representing 100 sweet oranges and 100 mandarins. For RT-qPCR, the RNA was used as a template using a fluorescently labeled minor groove binding qPCR probe for the detection of CPsV. The RNA extraction technique for the citrus tissues was optimized and the quality of RNA extraction was confirmed by targeting the internal control gene i.e. cytochrome oxidase to confirm the presence or absence of CPsV in the germplasm from Pakistan. Results from both the RT-PCR and RT-qPCR showed that no infection was present in the samples. The molecular methods described could be used in citrus certification programs and to test trees in nurseries and commercial orchards of Pakistan.

Keywords: Cox internal control; Fluorescently labeled probe; Pakistan; Psorosis; Taqman RT-qPCR

Introduction

Citrus fruit is one of the highest well-liked fruit crops of the human beings. More than 140 countries in the world are cultivating the fruit in its various forms and groups due to its increased usage in food as well as fresh fruit juice. The common groups of citrus grown worldwide are mandarin, sweet oranges, kumquats, grapefruits, lemons and limes (Su, 2008). At present the world's citrus production is about 136 million tonnes on an area of about 9678766 ha yielding about 140267 kg/ha in the year 2013. Islamic Republic of Pakistan occupies 14th position among the renowned citrus fruit producing countries for the year 2013. Pakistan's citrus production is about 215 million tons on an area of about 195300 ha yielding about 110087 kg/ha in the year 2013 (FAO, 2013) and Pakistan has a total share of 2% in world citrus production (Selli *et al.*, 2004).

Unfortunately citrus plants are facing many troubles regarding the various pathogens attacking the crop. The variety of pathogens include the bacterial (HLB, Canker), fungal (Scab), viral (CTV, CPsV) and many others (Atta *et al*., 2012). Among the viral pathogens, *Citrus psorosis virus* (CPsV) is the oldest and utmost damaging virus disease of citrus that has been studied and researched since the start of 19th century after *Citrus tristeza virus* (CTV) (Roistacher, 1993). Psorosis is a widespread graft-transmissible viral disease in old citrus trees especially in the sweet oranges varieties. The disease expansion in the citrus is time-consuming and it may take several years to notify the symptoms such as bark scaling of the trunk, chlorotic lesions and yellow spots on the leaves. Gum may accumulate below the bark scales and may impregnate the xylem producing wood discoloration and vessel like stemming. These symptoms have been used for field diagnosis of psorosis as a disease of citrus showing symptoms of bark scaling in trunks and branches of mandarin, grapefruit and sweet oranges (Zanek *et al.*, 2008; Achachi *et al*., 2014).

Psorosis has gained attention globally as it is one of deadly citrus disease that is caused by infectious filamentous ophiovirus, CPsV, which is a tripartite virus having three single-stranded RNAs of negative polarity and has been inadvertently spread to most citrus growing areas through the movement of citrus propagative material (Ongania *et al*., 2003; Martin *et al*., 2004). The tools used to detect CPsV in citrus trees are mainly based on using indicator plants and on laboratory tests (Lin *et al*., 2000; Salomone *et al*., 2004) including enzyme-linked immunosorbent assay (ELISA) (Garcia *et al*., 1997; Nikolaeva *et al*., 1998; Zanek *et al*., 2006). These methods have many downsides such as the time necessary for symptom development on indicator hosts, the cost of growing and maintaining the indicator

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plants, the antigenic properties of the pathogens to be detected and variations in the sensitivity as well as the accuracy of the tests (Sambade *et al*., 2003).

Significant work has been made during the last 20 years to develop a more reliable, less expensive, and more sensitive molecular methods (Olmos *et al*., 1999), including reverse transcription–polymerase chain reaction (RT-PCR) (Rosa *et al*., 2007) and more recently real-time RT-PCR (Osman and Rowhani, 2006; Osman *et al*., 2015). Today, due to the availability of increasing numbers of genomic sequences of virus in public databases, reliable systems are designed to detect CPsV by conventional RT-PCR and high throughput real-time RT-PCR. The objective of this research was to establish these systems in Pakistan and to screen the major citrus germplasm of Pakistan with CPsV as well as to compare the results of both the molecular methods simultaneously.

Materials and Methods

Sample Collection

Citrus psorosis virus (CPsV) symptomatic and asymptomatic leaf samples shown in Fig. 1 were collected from the two major groups of citrus including Mandarin (Kinnow, Low seeded Kinnow, Feutral's Early, Dancy and Nagpurisangtra) and Sweet Oranges (Musambi, Red Blood, Jaffa, Valencia Late, Moro Blood, Westin, Tarraco, Tarraco-N, Pineapple and Kozan) were obtained in the orchards of Citrus Research Institute, Sargodha (Pakistan) to check the prevalence of CPsV. For each sample, at least 50 symptomatic and asymptomatic leaves were collected in labeled polythene bags and transferred to ice box until shifted to the laboratory at -20ºC.

Total RNA Isolation

Total RNA was extracted from the phloem containing midrib tissue of the leaves along with the petiole. 0.2 g of the plant tissues were homogenized in the equal volume of the extraction buffer I (100 m*M* NaOAc, 10 m*M* EDTA, 250 m*M* NaCl, 1% triton 100X) and extraction buffer II (Acidic Phenol pH: 4.0) using a chilled pestle and mortar. The homogenized samples were kept at room temperature for 5 min and then centrifuged at 12000 rpm for 5 min. The supernatant was transferred to the new tubes containing 3M KOAc and added three-column volume of chilled absolute ethanol and centrifuged at 12000 rpm for 10 min. After this the supernatant was discarded and the RNA pellet was washed with 70% ethanol. The total RNA was suspended in nuclease free water (Thermo scientific). Estimation of RNA yield and quality was done with a NanoDrop ND-1000 Spectrophotometer (OPTIZEN NanoQ) by measuring optical density at 260 nm and the ratio of the absorbance at 260 and 280 nm, respectively. The quality of RNA was also determined using 37% formaldehyde denaturing agarose gel electrophoresis. The total RNA after extraction was shifted to -80°C immediately or converted to cDNA (Green and Smabrook, 2012).

DNAase Treatment

The total RNA extracted have the presence of genomic DNA contamination. DNAase I (Thermo Scientific) was used to remove genomic DNA from RNA samples as it digests single or double stranded DNA.

RT-PCR and Real Time RT-PCR Primers and Probes

The primers specific to the CP gene location based on GenBank accession number AF036926 were used to detect the CPsV isolate in Pakistani citrus plants. The primers for the RT-PCR were previously reported by Rosa *et al.* (2007) and for RT-qPCR primer/probes were reported by Osman *et al*. (2015). The sequence and amplicon size of the primers and probe for RT-PCR and qPCRis given in Table 1.

RT-PCR

The complimentary DNA (cDNA) synthesis was done using 3ul of the total RNA (500 ng/μL concentration), 100 uM antisense primer, 10 m*M* dNTP's (Thermo scientific), 5X RT buffer (Thermo scientific), 0.1 M DDT and MMLV-RT (Thermo scientific). The tubes were then incubated at 70°C for 10 min, 37°C for 5 min, 42°C for 60 min and 70°C for 7 min in a thermal cycler in two steps. The cDNA was used as a template for the PCR and was stored at -20°C prior to use.

Conventional PCR

The Dream Taq Green Master Mix (Thermo scientific) was used for the conventional PCR assay. A 3 μL of the cDNA template was used in a 25 μL of the PCR reaction following the procedure described by Rosa *et al*. (2007). A PCR was performed using the following conditions: one cycle at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 58°C for 60 sec and 72°C for 60 sec, followed by one cycle at 72°C for 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide. Expected amplicon size was produced at 411 bp without any primer dimers.

RT-qPCR

For the detection of CPsV, RT-qPCR assay described by Osman *et al*. (2015) was used. The reaction setup and primer/probe for the COX assay, which works as internal control as well as for the CPsV RT-qPCR, were performed at Vidalikis Lab, Plant Pathology Department, University of California, Riverside, California, USA.

Table 1: Sequence of forward reverse primers and probes used for RT-PCR and RT-qPCR

S. No.	PCR type	Primer/Probe	Sequence 5'-3'	Expected amplicon size (bp)	Primer location
1	Real-time RT-PCR	COX- F	AATCTGACCTTCTTTCCCATGC	162	32-53
		COX- R	AAGTGATTGTTACGACCACGAAGA		194-171
		COX-**pFAM**	ATCCAGATGCTTACGCTGG		96-114
		CPsV- F1	TCACAAATCAGTGAGGAATTGAGC	154	792-816
		CPsV- F2	CACAAATCAGTGATGAATTGAGCC		793-817
		CPsV- R1	GCAAACCCAGCATATCTCACAG		947-925
		CPsV- R2	CGCAAACCCAGCATATCTTACAG		948-925
		CPsV- **pVIC**	TCTCAAGATTGATATAGACAAC		851-873
2	Conventional RT-PCR	CPsV-forward	ACAAAGAAATTCCCTGCAAGGG	411	Conserved region (Partial CP
		CPsV-reverse	AAGTTTCTATCATTCTGAAACCC		region from CPsV)

Fig. 1: Symptomatic and asymptomatic leaves for CPsV in the field

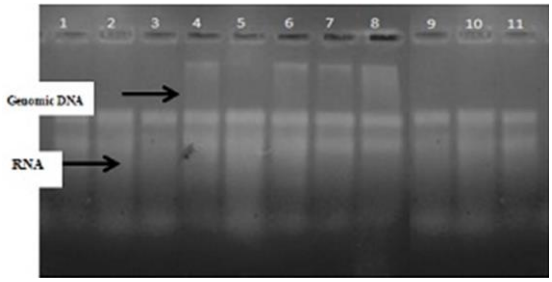

Fig. 2: Total RNA extraction from citrus tissues. Upper lane in figure shows presence of genomic DNA contamination in four samples which was then removed by DNAase treatment and lower lane shows presence of total RNA

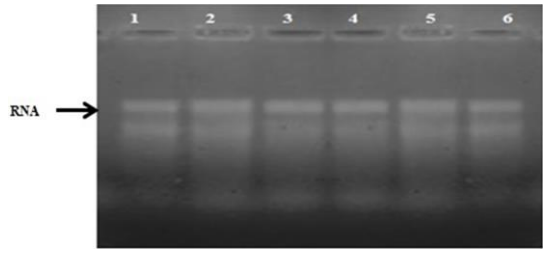

Fig. 3: DNAase treatment for the samples showing the presence of genomic DNA contamination in total RNA extraction

Results

Total RNA Extraction

Total RNA was extracted from both the symptomatic and asymptomatic citrus trees to check the presence of CPsV in the citrus plants. The above mentioned extraction procedure was used to isolate the total RNA from the petiole of the leaves. The procedure described above proved to yield a good quality of RNA that was proved by the NanoDrop analysis as well as 37% formaldehyde agarose gel electrophoresis (Fig. 2).

DNAase Treatment

RNA extracted from grounded samples have the presence of genomic DNA contamination. DNAase treatment was done to eliminate the contamination of genomic DNA. DNAase I (Thermo Scientific) and RNA free kit was used to remove genomic DNA from RNA samples as it digests single or double standed DNA. Integration and purity of RNA was then checked by running the samples on agarose gel electrophoresis in Fig. 3.

Detection of CPsV by RT-PCR

The presence of CPsV was confirmed by reverse transcription- polymerase chain reaction (RT-PCR). A 411 bp DNA fragment corresponding to the major coat protein gene of the virus was not amplified with any of the 200 samples (Fig. 4). Both the symptomatic as well as the asymptomatic samples gave the negative results. Positive control was amplified at 411 bp product size.

Detection of CPsV by RT-qPCR

A higher throughput and quantitative RT-qPCR with TaqMan primers and probes was evaluated and compared with standard RT-PCR for the detection of CPsV in same citrus samples. In this experiment, the RNA extracts from the same samples for the month of February were tested. The same results were observed from the samples and the positive control, negative control and non-template control worked perfectly. The quality of RNA extraction was checked before running the CPsV qPCR assay and all the samples were positive for COX internal control assay. The results for RT-qPCR assay for CPsV is shown in the Fig. 5. In general, the results were comparable in both tests, but real-time RT-PCR produced clearer results as positive control for CPsV, negative control for CPsV (Healthy Sweet

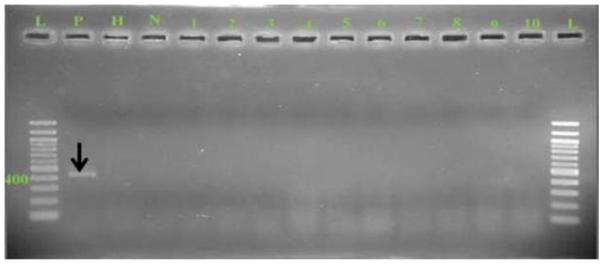

Fig. 4: RT-PCR results visualized on 1.5% agaorse gel electrophoresis. (Left to Right) L= 100 bp ladder, P= Positive control (Black arrow showing 411 bp), H= Nagative control (Healthy), N=Non-Template control, 1-10 = Pakistani samples

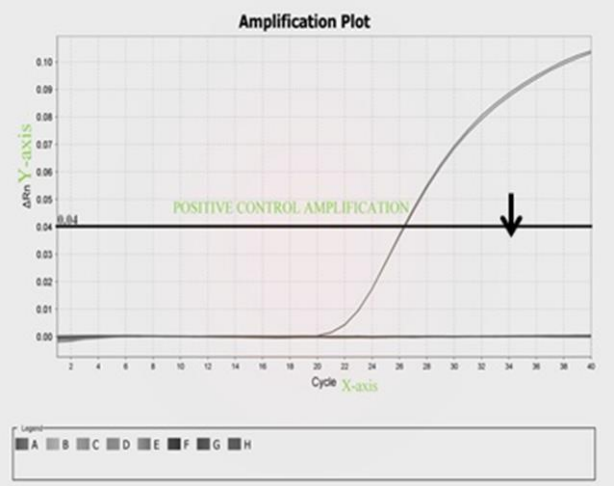

Fig. 5: Amplification plot of qPCR in singleplex RT-qPCR format. The X-axis displays the cycle number and they y-axis delta RN which shows the magnitude of the fluorescence single generated during the PCR at each time point

Orange) and non-template control were used during the same assay.

Discussion

In this paper, we account the RNA based molecular technique for the detection of CPsV for the citrus varieties cultivated in Pakistan. We used and compared the two molecular assay i.e., RT-PCR and RT-qPCR to find the advantages and disadvantages of both the methods. It has been reported that both the RT- PCR and real-time RT-PCR possess several advantages over one another but conclusively RT-qPCR is the most reliable molecular assay that can be used to diagnose the pathogen perfectly if present in the plants (Rosa *et al*., 2007; Osman *et al*., 2015).

Citrus has a distinctive position in the world among fruit crops but unfortunately different virus and viroid diseases are subsequently causing enormous yield losses and deteriorating the sensory properties of citrus fruits. Psorosis is one of the most severe and well-known viral disease that is associated with CPsV. The worldwide effective approach to control the spread of pathogens is to ensure that the propagating material used is virus-free (Bandyopadhyay and Frederiksen, 1999). But this approach itself is not sufficient for the pathogen control, appropriate identification and detection for all the possible pathogens of citrus is essential to overcome the problem.

During the present study, sampling was done on the basis of morphological symptoms found on the leaves of citrus that are considered as the first and basic step in identifying a disease including viruses. It has been reported earlier that virus can be present in symptomatic and asymptomatic citrus trees (Graham *et al*., 2013). The current study was also focused on the morphological characterization based on qualitative and quantitative of citrus cultivars belonging to sweet orange group showed low diversity among the cultivars. Most of the cultivars showed medium leaf and fruit shape and color are of commercial importance for citrus fruit marketing and trade (Nawaz *et al*., 2007).

The prime focus of the present study was to detect the CPsV in the citrus orchards of Punjab, Pakistan. So, a two-step RT-PCR assay was developed at the Plant Biotechnology Laboratory of LCWU. During the first step, extracted RNA was converted into cDNA and then cDNA samples were used as a template in conventional PCR during the second step. Singleplex and multiplex qPCR has been broadly used for the qualitative and quantitative screening of all the possible pathogens (Rodriguez-Gamir *et al*., 2012) particularly viruses (Osman *et al*., 2015). More recently, multiplex RT-qPCR have been reported to be as robust as singleplex RT-qPCR in detecting a broad range of different plant pathogens including citrus viral pathogens (Saponari *et al*., 2013).

The RT-PCR work done at LCWU lab showed negative PCR results, which was considered that it might be due to low virus titers. It has been reported earlier that low virus titers result in negative results of PCR. Garcia *et al*. (1997) and Rosa *et al*. (2007) also suggested that both the ELISA and RT-PCR methods can be used for detection of CPsV. Based on the above, it seems plausible to conclude that no experimental evidence was obtained of CPsV in sweet oranges as reported by D'Onghia *et al*. (2001) for their tested samples. The findings of Roistacher (1993) are in accordance with our work in which no virus was detected in citrus groups. The most distinguishable symptom of psorosis virus is bark scaling and that was not observed in any of citrus trees of CRI thus indicating virus may not be prevalent in the area.

The results of the above-mentioned work was then repeated at the University of California, Riverside Plant

Pathology lab for the same samples for RT-qPCR assay TaqMan technology and it was found that both the results were similar i.e., the samples were negative for CPsV. The work was also done and compared for both the assay for CPsV by Rosa *et al*. (2007) and Osman *et al*. (2015). RT-qPCR could effectively replace conventional RT-PCR in citrus diagnostics and such high throughput procedures can allow scrutinizing of the overall clean status of citrus trees (Vidalakis *et al*., 2010a, b).

The above findings can be narrowed down to the fact that nutrient deficient soils may lead to symptoms like chlorotic flecking, yellow ringspots and leaf variegation as the symptoms were observed during the present work. Reuther (1989), reported that nutrient deficient soils might lead to appearance of disease like symptoms that may mislead to the disease prevalence. However the high throughput molecular analysis has shown that the virus is not present in the samples collected from CRI, Sargodha, Pakistan.

Conclusion

The present study was significant in this regard that no published data is available on the detection of CPsV in Pakistan. The results of the current study may lead to the conclusion that this particular virus has no or very low prevalence in Pakistan. The symptoms may appear after few years but for now, no obvious symptoms of CPsV were observed in citrus fields. This is a good indication as the disease has catastrophic effects worldwide. However, further research is required to make sure the establishment of disease free clean plant material.

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