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Shelly Kapoor
Plant Virology Laboratory, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Anil Handa
Plant Virology Laboratory, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

Correspondence
Anil Handa
Plant Virology Laboratory, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India

RT-PCR based detection and molecular characterization of Prunus necrotic ring spot virus (PNRSV) in peach

Shelly Kapoor and Anil Handa

Abstract
Prunus necrotic ring spot virus (PNRSV) is an important virus of worldwide occurrence in stone fruits. It is a complex *Ilarvirus* consisting of five RNA components viz., RNA1, RNA2, RNA3, RNA4 and RNA5. Surveys conducted in four major peach growing districts of Himachal Pradesh revealed its occurrence on the basis of visual symptoms. Since PNRSV is a latent virus DAS-ELISA was further used to confirm its presence in most of the symptomatic trees. Subjecting the test samples to RT-PCR revealed the exact identity and resulted in molecular characterization of PNRSV in three trees of peach cv. *July Elberta* with highest concentration of PNRSV based on OD values obtained in DAS-ELISA. RNA3 component of 665bp size of PNRSV in coat protein (CP) was identified on 1.0 percent agarose gel. Cloning and transformation further confirmed the same RNA3 component in PNRSV in *E. coli* DH5α cells using PCR.

Keywords: PNRSV, DAS-ELISA, RNA, RT-PCR

Introduction
Peach diseases, particularly virus and virus like diseases have a major impact on peach production worldwide and often become dominant yield limiting factor in orchard management. Prunus necrotic ring spot virus (PNRSV) is one of the major viruses of stone fruits reported from different parts of the world (Fulton 1985; Brunt 1996; Kapoor and Handa 2017a; Kapoor *et al*. 2018a) [1-4]. PNRSV being a 'latent' virus rarely expresses itself on most commercial cultivars of stone fruits, but may cause specific symptoms on leaves of a few cultivars like chlorotic and necrotic shot holes and distortion of mid vein (Fulton 1985; Brunt 1996; Kapoor *et al*. 2018a) [1, 2, 4]. Based on symptomatology marked trees were subjected to DAS-ELISA to detect PNRSV in them (Kapoor and Handa 2017a; Kapoor *et al*. 2018b) [3, 5]. The exact identity of virus was established by using RT-PCR as PNRSV is a RNA virus consisting of five components viz., RNA1, RNA2, RNA3, RNA4 and RNA5 with each RNA having different role to play. Out of these, RNA3was done is largely responsible for virulence in stone fruits. Therefore, the isolation of RNA3 using RT-PCR and was subsequently confirmed on gel electrophoresis with a desired amplicon of 665bp (Mahfoudhi *et al.* 2015) [6]. Cloning and transformation was further used to confirm the desired amplicon of PNRSV using eTA vector construct incorporated in *E. coli* DH5α competent cells using colony PCR.

Materials and methods
Survey
Surveys were conducted during active growing seasons of 2015, 2016, 2017 and 2018 in different peach growing districts of Himachal Pradesh to record the occurrence, distribution and incidence of viral diseases in peach orchards situated at different locations using the formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of plants infected}}{\text{Total number of plants}} \times 100$$

Serological detection through DAS-ELISA

Alkaline phosphatase based direct double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to detect the virus as per the protocol of Clark and Adams (1977) [7] with slight modifications. The results of ELISA for detection were interpreted in accordance with Lemmetty (1988) [8] and Dijkstra and Jager (1998) [9] as samples were considered infected when their OD values at 405nm exceeded two times the mean values of respective healthy and negative control samples.

PCR based molecular characterization

In light of the fact that Prunus necrotic ring spot virus is one of the major latent viruses infecting peach, reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA extracted from leaves of three peach trees recording highest concentration of PNRSV in DAS-ELISA test to further establish the exact identity of the virus. The procedure proposed by Marbot *et al.* (2003) [10] was followed for PCR studies RT-PCR and PCR components and conditions were standardized and presented in Tables 1 and 2.

Table 1: RT-PCR reaction components

Components	Reaction volume	Final concentration
10X cDNA synthesis buffer	2.0 µl	1X
dNTP mix (10mM)	0.8 µl	2.5 mM
RNA Primer oligo dT (0.5 µg/ µl)	2.0 µl	1 µM
RT Enhancer (20U/ µl)	1.0 µl	20 U/ µl
Verso Enzyme mix(20U/ µl) (M-MuLV)	1.0 µl	20 U/ µl
Nuclease free water	3.2µl	-
Template (RNA)	10.0 µl	Upto 2 µg per 20 µl
Total	**20.0 µl**	

Table 2: RT-PCR cycle set up for cDNA synthesis

Process	Temperature	Duration	Number of cycle(s)
cDNA synthesis 1	25°C	10 minutes	1
cDNA synthesis 2	37°C	120 minutes	1
Inactivation	85°C	5 minutes	1

Translation elongation factor-2 (TEF2)

Peach-TEF2 F GGTGTGACGATGAAGAGTGATG and Peach-TEF2 R TGAAGGAGAGGGAAGGTGAAAG mRNA (a reference gene/housekeeping gene with accession number TC 3544) with product size of ~ 129 bp was used as an internal standard to rule out the probability of false positive reaction (Zhaoguo *et al.* 2009) [11].

cDNA amplification by PCR

PCR assay was carried out for the amplification of cDNA strand according to the protocol given by (Sambrook and Russel 2001) [12] and was modified accordingly. CP gene primer pair PNRSV-10F (TTCTTGAAGGACCAACCGAGAGG) and PNRSV-10R (GCTAACGCAGGTAAGATTTCCAAGC) as mentioned by (Marbot *et al.* 2003) [10] was used to amplify a ~ 665 bp region of coat protein (CP) gene (Tables 3 and 4) and the desired amplicon was observed on 1.0 percent agarose gel electrophoresis.

Table 3: Components of PCR reaction mixture for amplification of PNRSV internal control and coat protein gene.

Components (stock concentration)	Reaction volume	Final concentration
cDNA	2.0 µl	2.0 µl
Forward Primer (PNRSV-10F)	0.5 µl	10 pM
Reverse Primer (PNRSV-10R)	0.5 µl	10 pM
10X Reaction Buffer	2.5 µl	1X
dNTP mix (10mM)	1.6 µl	2.5mM
Taq DNA polymerase (3U/µl)	0.5 µl	5U/ µl
Nuclease free water	12.4 µl	-
Total	20.0 µl	

Table 4: PCR cycle set up for amplification of PNRSV coat protein gene

Steps	Temperature (°C)	Duration (minutes)	No. of cycles
Initial Denaturation	94	2:00	
Denaturation	94	0:30	30
Annealing	59	1:00	
Extension	72	1:00	
Final Extension	72	10:00	

Cloning and transformation of the desired amplicon

The PCR product was fractionated on 1.0 percent agarose/ethidium bromide gel. The specific band was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The eluted CP gene product was further ligated into pDrive eTA vector consisting of genes for antibiotic resistance and multiple cloning sites (MCS) and cloned into competent *E. coli* cells. The competent cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russel (2001) [12] and were further transformed. Confirmation of putative transformed bacterial colonies was done through colony PCR. The desired amplicon was submitted for sequencing using Sanger sequencing (Sanger *et al*. 1977) [13].

Results

The present investigations were conducted to study the occurrence, distribution, identification and characterization of Prunus necrotic ring spot virus (PNRSV) in different peach growing areas of Himachal Pradesh. Attempts were made to detect PNRSV on the basis of serological and molecular techniques (DAS-ELISA and RT-PCR). Field surveys were conducted between 2015-2018 in 40 orchards representing major peach producing districts of Himachal Pradesh. Owing to its latent nature, symptoms of PNRSV are largely masked. However, some of the typical symptoms in several orchards in Solan, Sirmour, Shimla and Kullu districts were leaf damage in the form of chlorotic and necrotic shot holes, mid vein distortion Fig. 1) and the disease incidence was recorded in the range between 2 percent in Shimla to 37 percent in Sirmour district.

Fig 1: Symptomatic leaves of Peach

Serological detection of PNRSV through DAS-ELISA

DAS-ELISA was performed for the detection of PNRSV in the leaf samples drawn from symptomatic trees marked in the orchard (Fig. 2).

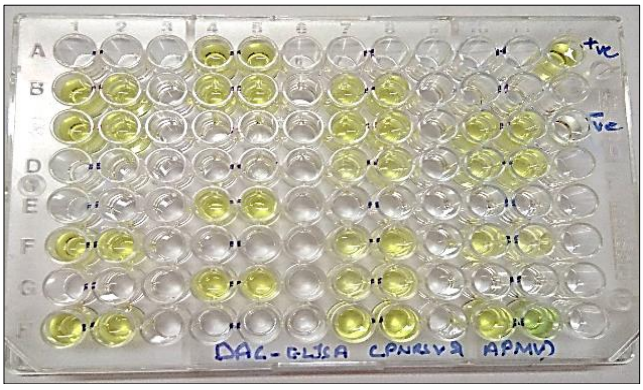

Fig 2: DAS-ELISA positives of PNRSV in peach

Molecular characterization of PNRSV

Total RNA was isolated from the symptomatic leaves of infected peach trees found positive in DAS-ELISA for PNRSV and cDNA was synthesized from isolated RNA. The amplification of ~ 129 bp of housekeeping gene product and desired amplicon of ~ 665 bp PNRSV coat protein gene product were obtained at annealing temperature of 59.9 °C for 1 minute with 30 cycles. PCR product was examined by electrophoresis on 0.8 per cent agarose gel. Sharp bands of ~129 bp of internal control and ~665 bp of PNRSV CP gene were obtained on the gel when compared with 100 bp marker ladder on lane M (Fig. 3).

Cloning and transformation of the desired amplicon

eTA cloning vector and *E. coli* DH5α competent cells tested on Luria Bertani Agar (LBA + Amp_{100}, X-gal, IPTG) were successfully transformed and Blue/White selection was done to screen out the transformed colonies. Further the detection of successful ligation of gene of interest in the vector using colony PCR was observed (Fig. 4) and clones confirmed through colony PCR have been submitted for sequencing.

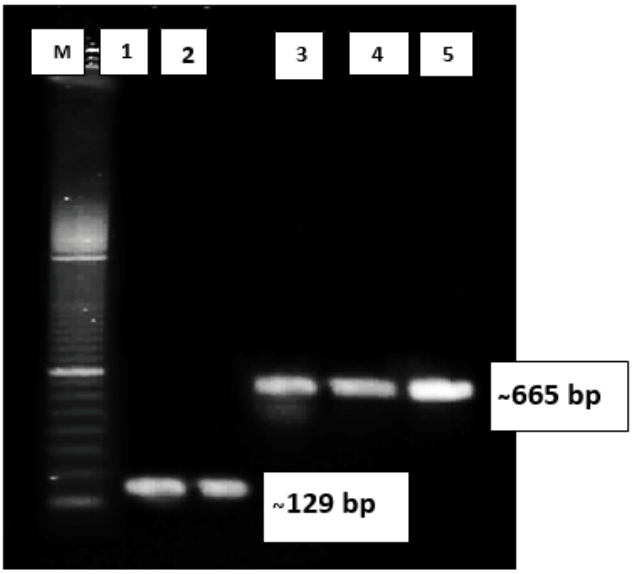

Fig 3: M (marker 100 bp), 1 & 2 (internal control Primers~129 bp), 3, 4 & 5 (PCR products~665 bp)

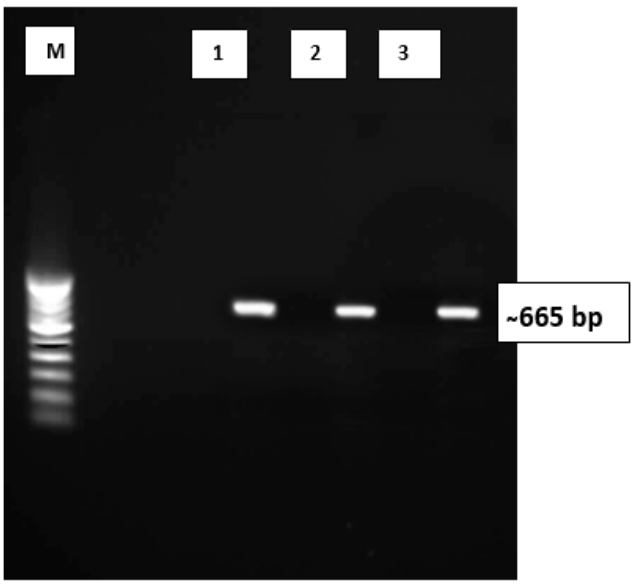

Fig 4: M (marker 100 bp), 1, 2 & 3 (Colony PCR products of RNA3~ 665 bp)

Discussions

Surveyed orchards showing chlorotic and necrotic shot holes and mid vein distortion in peach resulted to be the peculiar symptoms of PNRSV as these observations were similar with the reports of different workers with respect to PNRSV in stone fruits (Wells *et al.* 1986; Brunt *et al.* 1996; Hammond 2011; Winkowska *et al.* 2016; Kapoor and Handa 2017a) [14, 2, 15, 16, 3]. Incidence of PNRSV on peach has a huge impact worldwide as reported by different workers such as 60 percent in Georgia (Wells *et al.*1986) [14]; 25 percent in Turkey (Gumus *et al.* 2007) [17]; 70 and 100 percent in South Carolina (Scott 2014) [18]; 15 percent in Central Bohemia, Czech Republic (Winkowska *et al.* 2016) [16] and 18 percent in India (Kapoor and Handa 2017b) [19]. Use of DAS-ELISA for detecting viruses in peach is widely practiced and findings of the present studies are in line with those of a number of researchers who have observed DAS-ELISA to be an effective technique for detecting PNRSV in peach (Ghanem 2000; Almaraz *et al.* 2008; Chandel *et al.* 2013; Vemulapati *et al.* 2014; Kapoor and Handa 2017a; Kapoor *et al.* 2018b) [20, 21 22, 23, 3, 5]. There are specific reports on the use of RT-PCR for the detection of PNRSV in peach in support of the findings of present studies (Guo *et al.* 1995; Meisel *et al.* 2005; Milton *et al.* 2010; Herranz *et al.* 2013; Zindovic *et al.* 2015; Kapoor 2018) [24-29].

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