PRUNUS NECROTIC RINGSPOT ILAR VIRUS INCIDENCE IN APRICOT AND PEACH TREES IN SAUDI ARABIA

[13]

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ABSTRACT

Cultivated areas with fruit trees in Saudi Arabia are estimated at 233,513 h and produce more than 1.6 million tons of fruits annually. *Prunus necrotic ringpsot virus* (PNRSV), *Prune dowarf virus* (PDV) and *Plum pox virus* (PPV) are the most important and common viruses infecting stone fruit trees in nearby countries, Jordan, Lebanon, Syria and Egypt. In spring 2009, field surveys were carried out in an area of stone fruit production (Al Juof - North of Saudi Arabia) to record virus incidence of stone fruit trees. Apricots and peaches were observed showing chlorotic rings, necrotic spots, and a shothole appearance. A total of 166 leaf samples (65 Apricots and 101 Peach) were collected and transferred to the Lab. to be analyzed for the causal virus. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was performed using the commercially available for PNRSV. Result showed that 30 (11 Apricot and 19 Peach) out of 166 leaf samples were infected with PNRSV. Total RNA, from same samples used in ELISA test, was extracted in accordance with its instructions to confirm ELISA results. RT-PCR was performed using for PDV, PNRSV and PPV specific primers. RT-PCR resulted in the amplification of a 346 bp fragment as expected and negative result with PPV and PDV. The obtained result indicating the presence of PNRSV, in Apricot and Peach in Saudi Arabia, is recorded. The amplified fragment was sequenced and deposited in GeneBank (Accession No. HM584814). The sequence was compared with PNRSV isolates and had 100% identity with AF170170 from Czech Republic while 97% identity for PNRSV from USA (AF013287). Further investigations needed for other commercial orchards and nurseries. This result demonstrates first detection of PNRSV in Saudi Arabia.

Key words: ELISA, PNRSV, virus, detection, Saudi Arabia.

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INTRODUCTION

Cultivated areas with fruit trees in Saudi Arabia are estimated at 233,513 ha and produce more than 1.6 million tons of fruits annually **(Anonymous, 2010)**. *Prunus* spp. Are affected by many viruses and most frequently occur in the *Ilarvirus, Potyvirus* and *Trichovirus* **(Nemeth, 1986; and Jarosova and Kundu, 2010)**. *Prunus necrotic ringpsot virus* (PNRSV) is one of very important disease on stone fruit and present in nearby countries, Jordan, Lebanon, Syria and Egypt **(Myrta *et al.*, 2003; El Maghraby *et al.*, 2007 and Yousef *et al.*, 2002)**. PNRSV and Prune dwarf Virus (PDV) are the common and widespread in different stone fruits cousing crop losses **(Barbara, 1988 and Mink 1992)**, Currently these pathogen may be detected by biological indexing **(Bertozzi *et al.*, 2002 and Gentit, 2006)**, Immunological methods **(Mekuria *et al.*, 2003)**, molecular hybridization **(Palkovics *et al.*, 1994 and Saade *et al.*, 2000)** and reverse transcription polymerase chain reaction (RT-PCR) **(Wetzel *et al.*, 1991 and 1992; Scott *et al.*, 1992; Olmos *et al.*, 1997; Rosner *et al.*, 1998 and Spiegel *et al.*, 1999)**. In all these assays, usually one pathogen is detected per assay **(Hadidi *et al.*, 2004)**. Studies on harmful effects on the growth and yield of both viruses on various Prunus hosts have been published by a number of authors **Milusheva and Borisova (2005)**. **Paduch and Sala (2011)** were made a comparison studies between biological and molecular characteristics of three rose PNRSV isolates from Poland.

The present survey was conducted to estimate the incidence of PNRSV in stone fruit orchards and to investigate how the incidence of the virus varies among major cultivars and among production area.

MATERIALS &METHODS

Field surveys:

Field survey was carried out during spring 2007 and 2009 for the first time in stone fruit trees and nurseries growing in Al-Juof province North Saudi Arabia to determine the prevalence of stone fruit viruses. One hundred and sixty six leaf samples were collected randomly from orchards of apricot and peach. Samples were collected from symptomatic and symptomless shoots (Fig. 1). Samples were transferred to the laboratory for testing PNRSV occurrence using serological and RT-PCR techniques. The incidence of PNRSV infected trees was expressed as percentage.

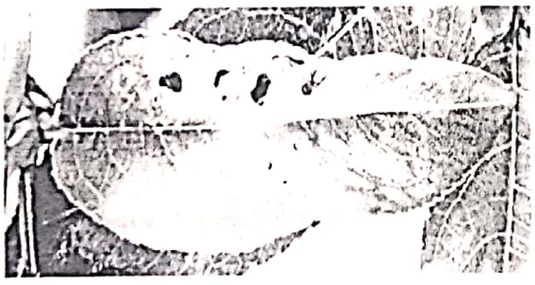

Figure 1. symptoms caused by PNRSV on peach leaves.

ELISA test:

Collected samples were tested for the presence of PNRSV by the double antibody sandwich of enzyme-linked immunosorbent assay (DAS-ELISA) as described by **Clark and Adams (1977)**, using commercially available IgG and Alkaline phosphatase-conjugated PNRSV IgG (Bioreba). ELISA readings were considered positive when the absorbance of was greater than at least twice the mean absorbance reading of two healthy control samples.

RNA isolation:

RNA was isolated from leaves of apricot and peach using a commercially available RNeasy plant mini extraction kit (QUAGEN Inc., CA) using the following protocol. Approximately 100 mg of fresh leaf tissue was pulverized 20 mg sodium metabisulphate and 1 ml of extraction buffer (RNeasy kit) containing 4% (w/v) PVP-40. Five hundreds of the homogenate was mixed with 60 µl of 20% (w/v) N-Lauroyl- sarcosine (Sigma) and incubated 70 °C with agitation for 10 min. Content was transferred to QIAshredder mini column and centrifuged at 14,000 rpm for 5 min. The flow-through was transferred to a new microcentrifuge tube and mixed with equal volume 100% ethanol and the protocol after that was completed according to the manufacturer's instruction of the kit. Extracted RNA was stored at -20°C till use for RT-PCR.

Primers:

Primer for PNRSV were designed by **Sanchez-Navarro *et al.*, (2005)** to amplify the partial of coat protein of RNA3, primers sequences of PDV were designed by **Youssef *et al.*, (2002)** were used to amplify 172 bp of coat protein of PDV and finally primers of PPV that designed by **Hadidi and levy (1994)** were used to amplify 220bp of nontranslated region

of the PPV viral genome as shown in table (1).

Table 1. Sequences of the oligonucleotide primers used for PCR amplification.

Virus	Primer name	Primer sequence	Product size	Reference
PNRSV	PNRSVr PNRSVf	5'GAACCTCCTTCCGATTTAG'3 5'GCTTCCCTAACGGGGCATCCAC'3	346 bp	Sanchez-Navarro *et al.*,2005
PDV	PDVr PDVf	5' TAGTGCAGGTTAACCAAAAGGAT'3 5' TGGATGGGATGGATAAAATAAT'3	172bp	Youssef *et al.*, 2002
PPV	PPVr PPVf	5'GTCTCTTGCACAAGAACTATAACC'3 5' TAGTGGTCTCGGTATCTATCATA'3	220bp	Hadidi and Levy 1994

*r = complimentary or reverse primer, f = Viral of forward primer.

Reverse transcription polymerase chain reaction (RT-PCR):

The total RNA from collected plant tissues according to the kit procedure, 10 µl of total extracted RNA were mixed with 25 pmol of the specific complementary primer as shown in table (1), then 3.5 µl free nuclease water was added to final volume of 15 µl. The mixture was heated to 70°C for 5 min. and then cooled on ice for 2 min. This mixture was used for reverse transcription at 37°C for one hr with 200 units of M-MLV reverse transcription (Promega, USA) in 1X RT buffer, 25 units of rRNasin ribonuclease inhibitor (Promega, USA) and 10 mmol of each of dATP, dTTP, dCTP and dGTP.

PCR was performed in 50µl total volume mixture containing 5µl of RT mixture (cDNA), 20 pmol of each forward and complimentary primers (as showed in table 1), 10 mmol of each of four dNTPs, 25µM of MgCl2, 2.5 units Taq polymerase (Promega, USA), and 5µl of 10X PCR buffer. PCR was carried out in the Biometra T-Gradient thermocycler with one initial denaturation cycle at 94°C for 2 min. followed by 35 cycles of amplification with temperature profiles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C. Thirty five cycles were applied followed by one final extension cycle at 72°C for 7 min. and the PCR amplified products were analyzed in 1.5% agarose gel, stained with ethidium bromide then scanned with UV illuminator.

DNA cloning and sequencing:

The amplified fragments of were ligated directly into pGEM-Teasy Vector (Promiga corporation, USA). The recombinant plasmids were introduced into *E. coli* strain DH5α as described by manufacturer's instructions. DNA was prepared from selected white colonies, digested with EcoRI and fractionated on agarose gel using 1Kb DNA ladder (Stratagene, Germany). Clones of

PNRSV having 346bp insert were selected for nucleotide sequencing.

The nucleotide sequencing was conducted under BigDye™ terminator cycling conditions and the reacted products was purified using ethanol precipitation and run using 3730XL automated DNA Sequencer (Macrogen Company, Seoul, North Korea) Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al.*, 2011) with those of PNRSV isolates available in GenBank as shown in table (2).

Table 2. Thirteen PNRSV isolates used in homology analysis.

No.	Country	Crop	GenBank accession No	Author
1	China	Rose	FJ610344	Shang
2	Czech Republic	Prunus domestica	AF170170	Vaskova
3	India	Wild cherry	AM493717	Chandel
4	NLM	NLM	S78312	Guo
5	Poland	Plum	DQ983495	Sala-Rejczak
6	Poland	Cherry	AF332615	Malinowski
7	Saudi Arabia	Peach	HM584814	Alhudaib
8	Spain	Malus	Y07568	Pallas
9	Turkey	Prunus persica	EF519311	Ulubas
10	USA	Prunus cerasus	FJ231737	Oliver
11	USA	Prune	AF013286	Scott
12	USA	Prune	AF013287	Scott
13	USA	Malus domestica	U15608	Berger

NLM. GenBank staff at the National Library of Medicine created this entry.

RESULTS

Field surveys and ELISA test: Survey was conducted in many orchards during spring 2007 and 2009 for the first time in stone fruit trees and nurseries growing in Al-Juof province North Saudi Arabia to determine the presence of stone fruit viruses *i.e.* PDV, PPV and PNRSV.

The result of ELISA test for collected samples of stone fruit with symptoms of infections from different orchards and Nurseries in Al-Juof, Saudi Arabia was recorded in table (3). The data of the ELISA test showed 26.9 % and 10.3% of apricot from orchards and Nurseries respectively have infection with PNRSV. While 24.5% and 12.5% of peach orchards and Nurseries

respectively have infection with were infected with PDV or PPV. PNRSV. No samples, in all cases,

Table 3. Virus detection by ELISA in Saudi Arabia-Al-Jouf on peach and apricot orchard and Nurseries.

Location AlJouf	Total no tested samples	PPV	PNRSV	PDV	Total No. of infected Samples	Infected %
Orchard A*	26	0	7	0	7	26.9
Nurseries A	39	0	4	0	4	10.3
Orchard P*	53	0	13	0	13	24.5
Nurseries P	48	0	6	0	6	12.5
Total	166	0	30	0	30	18

*A=Apricot, P=Peach.

Detection of PNRSV using RT-PCR:

RT-PCR was used to detect the viruses PPV, PDV and PNRSV using set of primers PPVr &PPVf, PDVr & PDVf and PNRSVr & PNRSVf (table1) respectively. PNRSV was detected in some tested samples using RT-PCR in both years 2007 and 2009. RT-PCR resulted in the amplification of 358 bp using the primers PNRSVr & PNRSVf in peach and apricot samples. However there are no bands obtained when using the specific primers for PPV and PDV as shown in Fig 2.

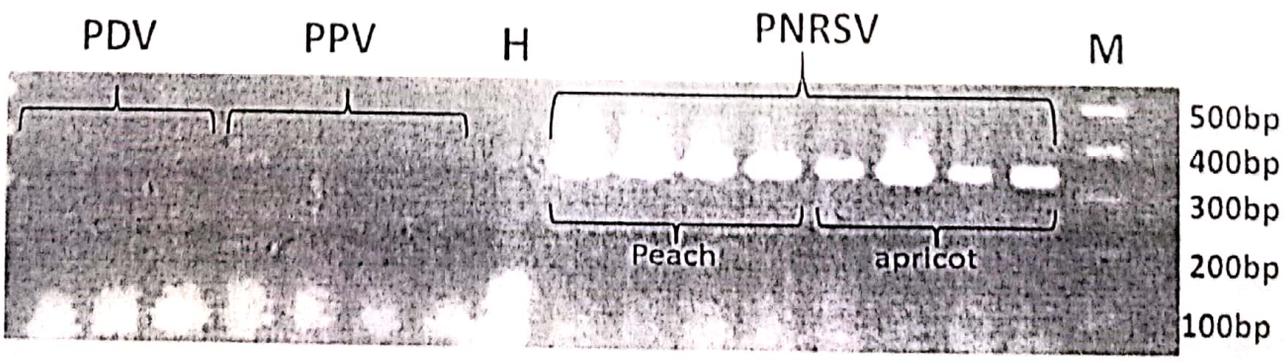

Figure 2. Gel electrophoresis of the RT-PCR assay for individual detection of PNRSV, PPV and PDV.

Nucleotide sequence and Phytogenetic analysis:

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The tree with the highest log likelihood (-507.4548) is shown (Fig. 3). The analysis involved 13 nucleotide sequences. The homologous sequences of apple mosaic virus (ApMV) were used for rooting purposes (accession number U15608). The present CP sequences were homologous to the sequences of PNRSV (S78312,AF013286 AM493717, DQ983495 , EF519311, AF170170 and FJ231737 100%), and other PNRSV (AF013286 99%, FJ610344 98%, Y07568 98%, AF332615 98% and AF013287 97%) in the NCBI databases.

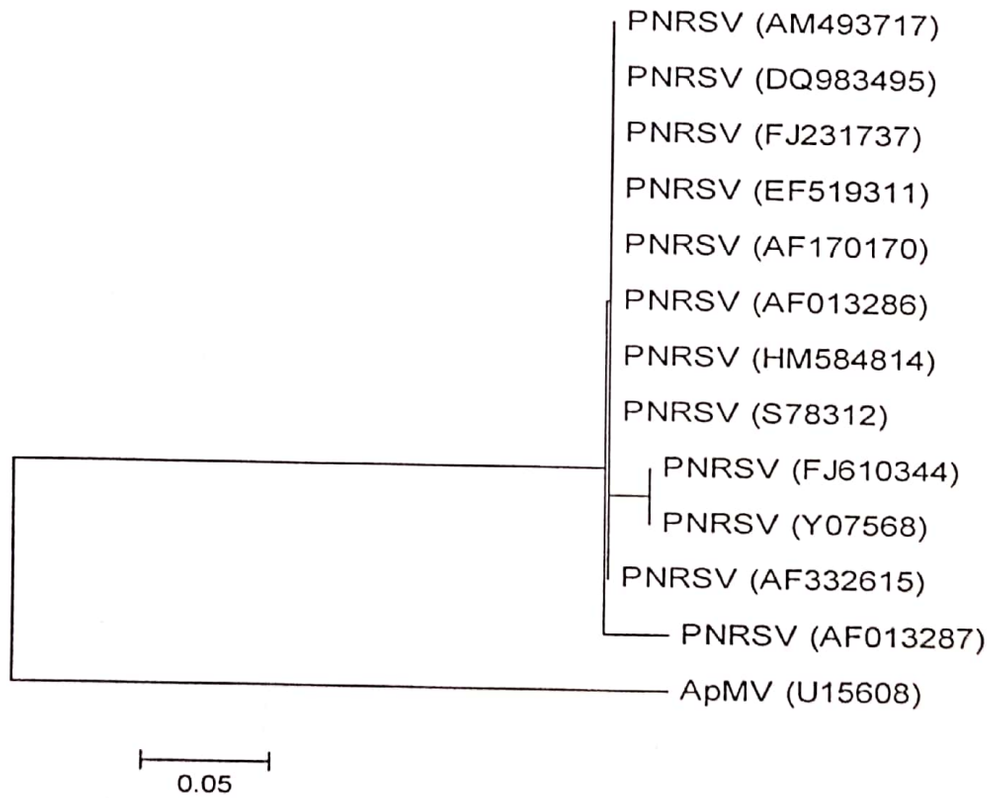

Figure 3. Phylogenetic trees of CP of PNRSV was performed with the 13 PNRSV isolates by using the Maximum Likelihood methodbased on the Jukes-Cantor model. The trees are rooted to the corresponding CP sequences of ApMV (Accession number U15608). The 0.05 bar indicates one nucleotide change per 100 nucleotides.

DISCUSSION

PNRSV incidence has been recorded, for the first time, in Saudi Arabia by molecular and serological assay by this work. The result revealed that PNRSV was found with high percent in apricot orchards and nurseries (16.9 %). Percentage of PNRSV occurrence in Peach orchards and nurseries was higher (18.8%) in Al-Jouf area. The result also revealed that PPV and PDV were not detected in the tested plants. RT-PCR amplification has only two samples were different however the ELISA technique detected PNRSV in 30 out of 166 samples of peach and apricot but RT-PCR technique detected 32 out of 166 samples (Table3). RT-PCR sensitivity is higher than ELISA. Obtained result agreed with results obtained by **Mekuria *et al.*, (2003)**. The detection of PNRSV by ELSA was shown by **Bertozzi *et al.*, (2002)** as more sensitive technique but when collecting the plant material in spring period. PNRSV was detected in leaves of infected almond trees by **Mekuria *et al.*, (2003)** using RT-PCR from early spring to late autumn. **Hassan *et al.*, (2006), and Jarosova and Kundu (2010)** were described protocol for multiplex of PCR in one tube system for detection PNRSV, PDV and PPV in stone fruit trees. Similar result were obtained by **Yardimci and Culal (2011)** when used ELSA and RT-PCR by to test the presence of PPV, PDV, PNRSV, ACLSV and ApMV. PNRSV, PDV and ACLSV but no PPV and ApMV were detected. They found that the RT-PCR is more sensitive than ELISA. This confirms our results. At the nucleic acid level, the CP genes of PNRSV shared 100% identity with those of CP-PNRSV ((S78312, AF013286k, AM493717, DQ983495, EF519311, AF170170 and FJ231737), but 99-97% identity with other CP genes of PNRSV (AF013286, FJ610344, Y07568, AF332615 and AF013287). This study presents the first report of PNRSV in Al-Jouf in North of Saudi Arabia. This virus was also recorded in most of neighbor countries such as Jordan, Lebanon, Syria and Egypt (**Jawhar *et al.*, 1996, Choueiri *et al.*, 2001, Myrta *et al.*, 2003, Salem *et al.*, 2004, El Maghraby *et al.*, 2007**). This study could lead the virus cross-border movement. Further investigations are needed for other commercial orchards and nurseries

in Saud Arabia for mapping the virus epidemiology.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support by Al- Jouf Agricultural Development Company (JADCO) and King Faisal University (KFU). Also we are grateful to Professor AE Aboul-Ata for critical reading of the manuscript and for his suggestions.

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