Biological, serological and partial molecular characterization of *Prunus necrotic ringspot virus* isolated from rose in Taif region, KSA

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ABSTRACT

An isolate of *Prunus necrotic ringspot virus* (PNRSV) was obtained from naturally infected rose plants collected from different rose farms in Taif, KSA exhibiting a wide range of foliar disease symptoms including necrotic spots, wavy lines, oak leaf pattern, leaf deformation, reduction in number and diameter of flowers , color breaking and necrotic spots on flower petals .The virus was biologically purified from single local lesion formed on *Chenopodium quinoa*. The isolated virus was identified on the basis of symptomatology, transmissibility, serological tests and molecular technique. The virus was identified serologically by direct ELISA, dot and tissue blotting immune-binding assay using authentic and induced antiserum for PNRSV.RT-PCR with specific PNRSV primer was used to confirm the obtained results. No amplified product was obtained from healthy control. The partial RNA3 movement protein product from PNRSV infected rose directly cloned using the TA cloning system. Nucleotide sequencing revealed the clones to be portion of PNRSV genome. These results indicate that the virus in rose is an isolate of PNRSV from Taif, KSA.

Key words:
Rose (Rose hybrid L.),*Prunus necrotic ringspot virus* (PNRSV),*Ilarvirus*, DAS-ELISA, Dot blotting immune-binding assay, RT-PCR, Nucleotide sequence.

INTRODUCTION

Rose (Rose hybrid L.) is an economically important crop in the industry and cut flower roses in the world. Viruses that infect rose plants are mostly belonging to the genera *Ilarvirus* and *Neepovirus*. *Prunus necrotic ringspot virus* (PNRSV) belongs to the genus *Ilarvirus* and the family *Bromoviridae*. PNRSV is the causal agent of many economically important diseases in most cultivated *Prunus* species worldwide. A part from almond, apricot, peach, plum, sour and sweet-cherry trees, roses are also attacked by this virus (Nemeth, 1986). PNRSV is the most commonly found rose virus and isolated in many rose growing regions (Moury *et al.*, 2001, Paduch – Cichal, 2003, Abou EL-ELa, 2006, Abdel-salam *et al*., 2008). Some field grown rose cultivars show no symptoms of PNRSV infection, whereas others developed, line patterns, necrotic ring spot or yellow net on leaves, color breaking and streaked petals (Thomas, 1984, Curtis and Moran, 1986; Wong *et al*., 1988; Khaled *et al.*, 2013; Paduch-Cichal and Sala-Rejczak ,2011). PNRSV is transmitted by grafting in woody plants and mechanically transmitted by several herbaceous plants. PNRSV has a tripartite genome, where RNA1 and RNA2 encode proteins involved in viral replication and RNA3 encodes the 3a (putative movement protein, MP) and the 3b proteins (coat protein,CP) (Fauquet *et al*.,2005)

The purpose of this investigation is to determinate the natural incidence of PNRSV on rose plants in Taif region, KSA by symptomatolgy, serology and molecular techniques.

MATERIALS AND METHODS

Virus source and symptoms:

Samples from naturally infected rose (Rosa hybrid L.) plants suspected to be *Prunus necrotic ringspot* symptoms showing necrotic spots, wavy lines, oak leaf pattern ,reduction in number and

diameter of flowers , color breaking and necrotic spots on flower petals were collected from different rose farms in Taif, KSA. The isolate understudy was serologically identified using an authentic antiserum for PNRSV (SANOFI, Sante animal, Paris, France).

Virus isolation and propagation

Naturally infected rose leaf tissues which reacted positively with DAS-ELISA were used as a source of PNRSV and then mechanically transferred onto the tested plants. About 2g of naturally infected leaf tissues were ground in 4 ml of buffer 0.01 ml phosphate buffer, pH 7.4 according to Abdel-salam *et al.* (2008) then inoculated onto carborandom dusted leaves. *Chenopodium quinoa* was used as a local lesion host. The inoculated seedlings were kept in the greenhouse and were observed for symptoms development. The virus was purified biologically through three consecutive passages onto the local lesion host according to Kuhn (1964).

Serological detection of PNRSV

Serological tests of dot-blotting immune-binding assay (DBIA) and tissue blotting immune-binding assay (TBIA) (Lin *et al.,* 1990 and Abdel-salam .,1999) using authentic and produced polyclonal antisera against some viruses (Arabis mosaic virus (ArMV), Strawberry latent ringspot virus (SLRSV) and (PNRSV) from which affecting rose. Samples reacted positively with PNRSV were used for the subsequent experiments. Direct enzyme linked immune-sorbent assay (DAS-ELISA) (Clark and Adams,1977) was used for virus detection.

Molecular detection of PNRSV

Molecular tests were performed by the reverse transcription-polymerase chain reaction (RT-PCR) for the presence of PNRSV in rose plants.

Nucleic acid extraction and analyses

Total RNA was extracted from symptomatic and asymptomatic rose leaves as described in Plant Total RNA Mini Kit instruction manual (RBC Labs, Inc). 100 mg of frozen plant tissue was ground under liquid nitrogen to a fine powder, transferred to microfuge tube with 500µl of lysis buffer and incubated for 5 min at room temperature. The mixture was applied to the filter column, centrifuged for 2 min at 14,000 rpm, the filter column discarded and the clarified filtrate was transferred to RB column. Half of the volume of ethanol was added and centrifuged for 2 min at 14,000 rpm followed by washing twice using wash buffer and the column was dried. The total RNA was eluted by using 50µl of RNase-free water.

One Step RT-PCR Reaction:

The reverse transcription (RT) and PCR assay was performed in DNA engine thermal cycler (BioRad) using specific PCR primers for PNRSV shown in table 1 (Hammond *et al.*,1995). The reaction mixture was set up according to Verso ™1-Step RT-PCR Kit (Thermo Scientific, Inc). The mixture contained 2µl of total RNA extract, 12.5µl 1-Step PCR master mix (2X), 0.5µl of each PNRSV movement protein (RNA-3) primer (10µM), 1.5µl RT enhancer, 0.5µl Verso Enzyme Mix and sterile deionized water to a final volume of 25µl. The cycling parameters were: reverse transcription at 50°Cfor 15 min, verso inactivation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s. The final extension step was 72°C for 7 min. RT-PCR product was separated on 1% agarose gel electrophoresis, stained with ethidium bromide and visualized using BioRad Gel documentation system.

Table 1. Primers used for (PCR) for the detection of PNRSV.

virus	Sequence	bp
PNRSV-H	AGACGTCGTGACAGACGTCGAAG	300
PNRSV-C	TTCTGTACCTGCCAATATCCTACT TCG	

Cloning and sequencing:

The 300 bp rose PNRSV amplification product was isolated from ethidium bromide stained agarose gel using the crush and soak method (Maniatis *et al*., 1982). Purified products were directly cloned utilizing the single A overhangs left by Taq polymerase using the TA Cloning system (Invitrogen) according to the manufacturer's suggestions.

RESULTS

Virus source and symptoms

The virus under study was isolated from naturally infected rose plants collected from different rose farms in Taif, KSA. Infected leaves with PNRSV showed symptoms of necrotic spots, wavy lines, and oak leaf pattern. Whereas infected rose flowers exhibited leaf deformation , reduction in number and diameter of flowers, color breaking and necrotic spots on flower petals (Fig. 1-A &B).

Virus isolation and propagation

Naturally infected rose plants which reacted positively with PNRSV-specific antiserum using DAS-ELISA was used as a source for PNRSV. The virus isolate was mechanically transferred onto *chenopodium quinoa* seedlings and kept in the greenhouse. Systemic symptoms were appeared 2-3 weeks post inoculation. Inoculated *chenopodium quinoa* leaves showed systemic numerous chlorotic local lesions (Fig.2). On the other hand, inoculated rose plants by PNRSV showed symptoms of line patterns, ringspots which later changed to shoot holes afterwards and/or yellow nets on the leaves (Fig.3).

Serological detection of PNRSV

Serological detection of PNRSV was carried out in naturally infected rose plants collected from the field and thereafter in mechanically inoculated plants. All of the PNRSV infected rose samples were reacted positively with PNRSV-specific antiserum using DAS-ELISA. On the other hand, techniques of DBIA and TBIA on nitrocellulose membranes could be readily applied for detection of PNRSV in infected rose tissues under field and later under laboratory or greenhouse conditions. Result in Fig. (4) showed the efficiency of DBIA and TBIA for detection of PNRSV. Positive reaction was obtained as strong purple color.

Fig.(1-A): Symptoms of an infected rose plant with PNRSV in a commercial rose field in Taif governorate ,KSA showing necrotic spots, wavy lines, oak leaf pattern, reduction in number and diameter of flowers.

Fig.(1-B):PNRSV Symptoms showing color breaking and necrotic spots on flower petals.

Fig.(2): PNRSV symptoms developed on mechanically inoculated *chenopodium quinoa* seedlings showed systemic numerous chlorotic local lesions.

Fig.(3): Mechanically inoculated rose plants showed symptoms of-line patterns ,ring spots which later changed to shoot holes.

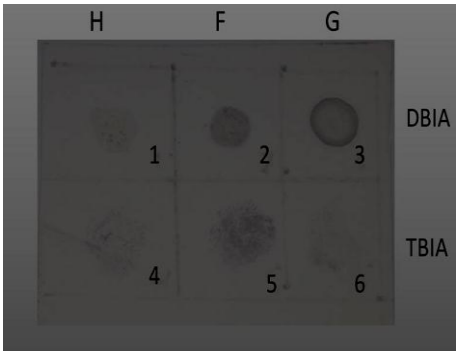

Fig.(4): PNRSV was detected by DBIA&TBIA tests using authentic polyclonal antiserum: (H1,4) healthy plant ,(F2,5) naturally infected rose plant ,and (G3,6) artificially infected rose plants maintained in the greenhouse.

Molecular detection of PNRSV

Total nucleic acid extracted from rose plants were subjected to RT-PCR analysis using conditions and primer previously reported. A specific product of about 300 base-pair (bp), encodes for RNA 3 movement protein from PNRSV was clearly visualized in the ethidium bromide stained agarose gel (Fig.5). Results of a RT-PCR assay were performed on samples taken from two infected rose plants, naturally and artificially, in addition to healthy plant as a negative control. No specific amplification was obtained with RNA extracted from healthy rose plant. In order to gain a better understanding of the molecular variability of PNRSV, PCR fragment derived from rose plant was cloned and sequenced.

Sequence analysis

Sequence analysis of cloned PCR product obtained from rose sample revealed that the positive signal was composed of PNRSV sequence (Fig.6). This sequence shares about 99% homology with the PNRSV isolate accession number EU368738 (Paduch-Cichal and Sala-Rejczak *et al.*, 2007) obtained from the GenBank. In addition, analysis also showed that the virus shares about 98% homology with the group of isolates with accession numbers AJ133208 (Aparicio *et al*., 1999), JX569828 (Zindovic *et al*., 2013), KF135205 (Cui *et al*., 2013) and JQ005037 (Cui *et al*., 2012) obtained from the GenBank. A resulted sequence was submitted to the GenBank and gets the accession number (**LC025530**) to the PNRSV isolated from Rose in Taif KSA. Phylogenetic tree was constructed from the multiple sequence alignment (Fig.7).

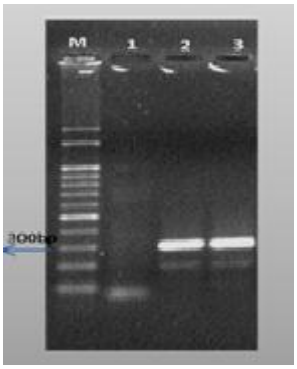

Fig.(5): Reverse transcription–polymerase chain reaction (RT-PCR) detection of *Prunus necrotic ringspot virus*, Lane M Sharp DNA ladder Marker 100bp (RBC), lane 1 Healthy rose plant sample as a negative control, lane 2 and lane 3 PNRSV isolated from natural and artificial inoculation, respectively. Amplified products were detected by Gel documentation system (BioRad) of ethidium bromide stained agarose gel following electrophoresis.

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NRSV      CGAATGGTTGGATTGGGATGGTGGAGGACTATAAGGTGGC    40
J133208   ----------------------------------------a   40
U368738   ----------------------------------------   40
Q005037   ----------------------------------c-------a  40
X569828   ----------------------------------------a   40
F135205   ----------------------------------------a   40

NRSV      ACAACCTGATGGTCCGAATGCCCTGTCTAGGAAGGGTTTC    80
J133208   ----------------------------------------a---  80
U368738   ----------------------------------------   80
Q005037   ----------------------------------------   80
X569828   ----------------------------------------   80
F135205   ----------------------------------------   80

NRSV      TTGAAAGACCAACCGAGAGGTTGGCAGTTCGAACCTCCCT    120
J133208   -----g----------------------------------t-  120
U368738   ----------------------------------------   120
Q005037   -----g----------------------------------t-  120
X569828   -----g----------------------------------t-  120
F135205   -----g----------------------------------t-  120

NRSV      CCGATTTAGATTTCGACACTTTTGCGCGTACGCATGTGT     160
J133208   ----------------------------------------   160
U368738   ----------------------------------------   160
Q005037   ----------------------------------------   160
X569828   ----------------------------------------   160
F135205   ----------------------------------------   160

NRSV      CGTTATCGAATTCAAGACCGAAGTGCCCGCTGGGGCCAAG    200
J133208   ----------------------------------------   200
U368738   ----------------------------------------   200
Q005037   ----------------------------------------   200
X569828   ----------------------------------------   200
F135205   ----------------------------------------   200

NRSV      GTCTTGGTTAGGGATTTGTACGTAGTGGTAAGTGATTAC     240
J133208   ----------------------------------------   240
U368738   ----------------------------------------   240
Q005037   ----------------------------------------   240
X569828   ----------------------------------------   240
F135205   ----------------------------------------   240

NRSV      CACGAGTGCAAATTCCGACTGATGTCTTGCTGGTCGAGAC    280
J133208   ----------------------------------tga      280
U368738   ----------------------------------tga      280
Q005037   ----------------------------------tga      280
X569828   ----------------------------------tga      280
F135205   ----------------------------------tga      280

NRSV      AGACCTGCTTG                                 291
J133208   ----------                                  291
U368738   ----------                                  291
Q005037   ----t-----                                  291
X569828   ----------                                  291
F135205   ----------                                  291
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Fig.(6): Nucleotide sequence alignment of the partial movement protein of PNRSV isolated from rose plants in Taif-KSA. Reference isolates with accession numbers (AJ133208, EU368738, JQ005037, JX569828, and KF135205) were collected from the GenBank and analyzed using DNAMAN program. Dashes indicate identical residues. Liter indicates no similarity.

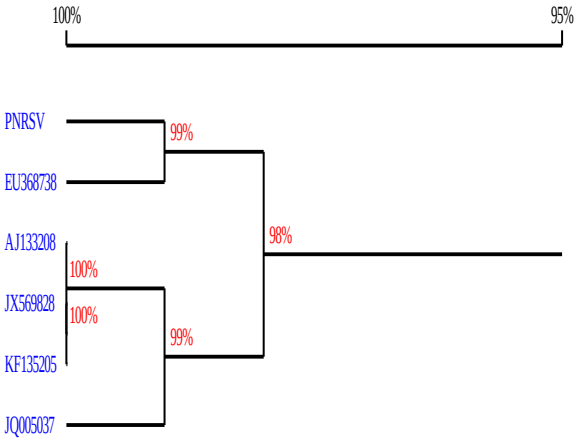

Fig.(7): Consensus phylogenetic tree constructed from the multiple sequence alignment of several major PNRSV isolates. Values at the nodes indicate significance in a boot strap analysis with 100 replicates.

DISCUSSION

Symptoms of different intensity appeared on foliage of all virus-inoculated roses: chlorotic yellow line patterns or chlorotic ringspot, yellow line patterns. These leaf symptoms were similar to those described earlier (Moran *et al*. 1988, Manners 1997; Moury *et al*. 2000; Paduch-Cichal 2003).

In this investigation PNRSV was isolated from naturally infected rose plants exhibited a wild range of foliar disease symptoms including necrotic spots, wavy lines, and oak leaf pattern, deformation, reduction in number and diameter of flowers , color breaking and necrotic spots on flower petals . On the other hand, the virus under investigation was easily transmitted mechanically to rose seedling in the greenhouse. Symptoms resulting from mechanical inoculation were similar to those observed in naturally infected plants. Such collected symptoms have previously been described for PNRSV

infection **(**Thomas1982 and 1984, Curtis and Moran 1986; Wong and horst 1988; Mansour, 2006; Paduch-Cichal and sala-Rejczak 2007; Abdel-salam *et al*, 2008).

Infected rose samples were brought to the laboratory for further detection at serological and molecular levels to confirm the identity of the virus isolate. PNRSV was serologically reactive to the authentic PNRSV-antiserum using direct ELISA. In addition, the induced antiserum was used to detect PNRSV in naturally infected rose in the field as well as in the greenhouse using the technique of DBIA and TBIA on nitrocellulose membranes. Immunological detection of viral antigens on nitro-cellulose membranes is very sensitive. We expect that the sensitivity of TBIA should be similar or equal to that of DBIA. One of the advantages of TBIA for virus detection is that can be prepared in any laboratory, greenhouse or even in the fields (Lin *et al* ., 1990). Such results are confirmed by several authors applying serological tests for PNRSV identification (Casper,1973 ;Barbara *et al*,1978; Barbara,1988;Moury ,2000; Abou EL-ELa, 2006; Abdel-Salam *et al*., 2008; Paduch-Cichal and Sala-Rejczak 2011 and khaled *et al*., 2013).

The presence of PNRSV in rose from Taif KSA has been confirmed in this work. Beside symptoms observed and the positive results by ELISA and either by TBIA or DBIA, it was possible to identify the virus by RT-PCR. The specific primers (Hammond *et al*., 1995) allowed obtaining amplicon of 300 bp of RNA3 for the PNRSV studied. In this work successfully used RT-PCR to detect PNRSV in total RNA isolated from infected rose leaves. This indicated the feasibility of RT-PCR as a rapid and accurate laboratory assay for detection of the virus under study.

Nucleotide sequence analysis of RT-PCR products obtained from rose plant confirmed its identity as an isolate of PNRSV as shown in the biological and serological assays. The highest similarity (99%) was found between the PNRSV-Taif isolate and the isolate from GenBank Acc. No. EU368738 (Paduch-Cichal and Sala-Rejczak *et al.*, 2007), followed by 98% with the isolates with accession numbers AJ133208 (Aparicio *et al*., 1999), JX569828 (Zindovic *et al*., 2013), KF135205 (Cui *et al*., 2013) and JQ005037 (Cui *et al*., 2012) obtained from the GenBank. Further studies could be important to sequence more isolates of PNRSV to monitor the viral genotypes and to able to follow possible changes in the virus population diversity.

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