Partial characterization of *Prunus Necrotic Ringspot Virus* on apple in EGYPT

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**ABSTRACT**

A severe isolate of *Prunus necrotic ringspot virus* (PNRSV) was isolated from apple orchards in the vicinity of Nubaria city, Beheira governorate, Egypt. Infected-apple trees showed chlorotic, necrotic ringspots, and shoot holes on leaves. Severely infected trees withered, became useless, and were removed causing severe economic losses. Reverse transcriptase (RT) polymerase chain reaction (PCR), RT-PCR, using degenerate primer pair for the coat protein (CP) gene of *I larvirus* amplified products similar to those produced from peach and apricot isolates of PNRSV-infecting stone fruits. Dot blotting immuno-binding assay (DBIA) showed positive reaction between PNRSV-infected apple sap and an Egyptian antiserum for PNRSV. Purified preparation from infected leaves, using the electro-elution technique yielded nucleoprotein which had A max and A min at 260 and 240 nm respectively. Electron microscopy examination showed spherical virions with ca. 26 nm in diameter.

**Key words:** *Prunus necrotic ringspot virus, Ilarvirus, RT-PCR, Dot blotting immuno-binding assay, Egypt*

**INTRODUCTION**

PNRSV belongs to the family *Bromoviridae*, genus *I larvirus* (isometric labile ringspot viruses) (Fulton, 1983) and includes many strains that differ in pathogenicity (Howell and Mink, 1988), biophysical (Crosslin and Mink, 1992) and serological properties (Spiegel et al., 1999). All genera of *Bromoviridae* including *I larvirus* contain tripartite genomes. The RNA1 and RNA2 code for proteins involved in viral replication and the RNA3 codes for both a movement protein and the viral coat protein (Murphy et al., 1995). These species of RNAs are encapsulated in isometric particles (23-27 nm in diameter) rounded in profile and without a conspicuous capsomere arrangement (Brunt et al., 1996).

PNRSV is graft, pollen and seed-transmitted (Gella, 1980, Uyemoto et al., 1992; Amari et al., 2004). The virus in most hosts induces shock symptoms after infecting plants, provided that they have not been infected earlier by latent strains of PNRSV.

PNRSV is the most common virus infecting *Prunus* species as peach and apricot (Mink, 1992, Myrata et al., 2003; Abdel-Salam et al., 2008a), rosaceous plants (Abdel-salam et al., 2008b), and naturally infecting other non-rosaceous plants (Abdel-Salam et al., 2006a). PNRSV is responsible for yield losses of up to 15% in sweet cherry and up to 100% in peach. PNRSV can reduce bud development in nurseries, decrease growth of fruit (10% to 30%) and fruit yield (20% to 60%), delay fruit maturity, and increase susceptibility to winter injuries in orchards (Oliver et al., 2009; Pallas et al., 2012).

In Egypt isolates of PNRSV were detected in peach and apricot grooves
(Abdel-Salam et al. 2008a), Rosa spp. (Abdel-Salam et al., 2008b) as well as on sugarbeet plantations (Abdel-Salam et al., 2006a). In the present study, incidence of PNRSV is reported on apple (Malus domestica). Severe symptoms mimic infections with PNRSV were recently detected on apple from several orchards in the vicinity of Nubaria city, Beheira governorate. Infected-apple samples were brought to the laboratory for further detection at serological and molecular levels to check the presence of virus. The present study reports the presence of an isolate of PNRSV on apple, viz. PNRSV-Apple.

MATERIALS AND METHODS

Virus isolates
An isolate of PNRSV was isolated from apple groves, Beheira governorate, Egypt. Infected samples showed chlorotic, necrotic ringspot, and shot holes. The virus isolate was purified biologically by mechanical inoculation on Chenopodium quinoa and Gomphrena globosa as described by Abdel–Salam et al. (1997, 2006a). Isolates for PNRSV from apple and apricot, preserved in the greenhouse facilities, Cairo University were used as positive controls.

Serologic studies
Dot blotting immunobinding assay (DBIA) test, described by (Abdel-Salam et al., 2008a) was used in measuring virus presence in tested hosts and serologic relationships between PNRSV isolates from apple, peach and apricot. Tissue samples were ground, filtered and diluted 1/10 in PBST buffer. An Egyptian antiserum for PNRSV prepared for the peach isolate (Abdel-Salam et al., 2008a) of the virus was used in the present study.

Virus purification
Fifty grams of fresh tissues of PNRSV-infected gomphrena (Gomphrena globosa) plants, previously inoculated with PNRSV-Apple, were used in virus purification. Purification of PNRSV-Apple utilized the electro-elution (EE) technique described by Abdel-Salam (1999).

The EE technique involved extraction of tissues (1:3 w/v) in 0.1 M NaH2PO4-Na2HPO4, pH 7.0, containing 1 mM EDTA, 20 mM Na2SO3, and 0.1% of each of 2-mercaptopethanol and thioglycolic acid. The extract was clarified with 12.5% volume of each of chloroform and butanol. The clarified-virus suspension was concentrated with 4% polyethylene glycol (4000, mw) and 1% NaCl. The concentrated virions were suspended in 1 mM phosphate buffer, pH 7.2, containing 1 mM EDTA (suspension buffer, SB). The virions were further purified with EE-ISCO tank with tank buffer containing 20 mM phosphate buffer, pH 7.2, and applying 4 mA/cell. The concentrated virions were then suspended in SB and measured spectrophotometrically.

Electron microscopy
Purified virus isolates were stained with 2% phosphotungestic acid, pH 7.2 according to Fulton (1981).

Genomic studies
Extraction of total RNA
Total RNA was extracted from PNRSV-infected peach and apricot plants by applying the silica-based technique described by Boom et al. (1990).

RT-PCR
The primer set CP (+) sense primer (5’ CCG AAT TTG CAA TCA TAC CCA CGT T 3’) and CP (-) antisense primer (5’ CGG AGA AAT TCG AGT GTG C 3’) complementary to the
conserved region of the coat protein (CP) gene were used to generate 704 bp fragments from PNRSV Cp gene (RNA-3) as described by Abdel-Salam et al. (2008). First strand cDNA was synthesized in a total of 20 μl reaction mixture. 5 μl of total RNA (~25 μg) was heated at 65°C for 8 min, chilled for 3 min in ice, then added to 15 μl reaction mixture containing 1.5 μl of antisense primer (10 pmol), 2 μl of M-MulV reverse transcriptase buffer (10X), 0.125 μl of M-MulV reverse transcriptase (5000 U), SibEnzyme Ltd, 2 μl dithiothreitol (100 mM), Promega, 1 μl dNTPs mix (10mM), 0.25 μl of ribonuclease inhibitor (40 U/μl), Promega, and 8.125 μl DEPC H2O. The mixture was incubated at 42°C for 1 h, incubated at 95°C for 3 min, and then kept in ice. PCR cocktail included 2 μl of the reverse transcription products, 5 μl of 5X Green GoTaq buffer, containing 1.5 mM Mg2Cl, Promega, 0.5 μl of dNTPs mix (10mM), 1 μl of each of sense and antisense CP primers (10 pmol, each), 0.25 μl of Go Taq polymerase (5 U/μl), Promega, 12.25 μl DEPC H2O. PCR conditions included 5 min at 95°C; followed by 35 cycles of amplification of 1 min at 95°C, 1 min at 53°C, 1 min at 72°C, and held for 10 min at 72°C. The RT-PCR amplicons were analyzed on 1% agarose at 100 V in 1/2 X TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide and examined with UV trans-illuminator.

RESULTS
Smptomatology
Primary symptoms on apple starts with leaf-chlorotic ringspot which turned necrotic afterwards (Fig.1-A). Shot hole symptoms followed the necrotic ringspot formation (Fig.1-B). Few small apple fruits are formed on infected tree (Fig. 1-C). Infected trees express shock symptoms (Fig.1-D) which extend to circumvent the whole tree and causing leaf defoliation (Fig. 1-E). Severely infected- trees wither out and carry no fruits (Fig. 1-E).

Serologic study
DBIA test was used to detect PNRSV-Apple isolate. Results in Figure (2) showed the positive reaction of sap from infected apple, apricot and peach with the induced antiserum for PNRSV-Peach isolate. The peach isolate of PNRSV reacted strongly with its homologous antiserum. While both isolates of apple and apricot of PNRSV reacted moderately with the antiserum of PNRSV-Peach indicating distant serologic relationship with the peach isolate of PNRSV.

Virus purification
The purified virus preparations, using EE technique, for PNRSV-Apple, had a UV spectrum typical to nucleoproteins with A max at 260 nm, A min at 240 nm, with A260/280 ratio of 1.6 (Fig. 3). Such results are typical to similar values reported for different isolates of PNRSV).
(e.g PNRSV (Abdel-Salam et al., 2006a, 2008a, b), some begomoviruses (Abdel-Salam, 1999, Abdel-Salam et al 2006b), an ipomovirus, and a crinivirus (Abdel-Salam, 2012). The EE technique is fast, low cost, and meets the demands of many moderately equipped laboratories.

Electron microscopy examination
Electron microscopy results of purified PNRSV showed spherical virions with an average diameter is 26 nm (Fig. 4).
Fig. (1): Symptoms of PNRSV infection on apple trees. A, development of chlorotic and necrotic ringspots; B, development of shoot holes symptoms; C, formation of small deformed fruits; D, development of shoot symptoms on leaves; E, extended shoot symptoms and severe leaf defoliation.

Fig. (2): DBIA showing the detection of PNRSV from infected apple, peach, and apricot trees using PNRSV antiserum prepared for PNRSV-peach isolate (at 1/1000 dilution in PBS buffer). Control represents healthy sap from apple. R1 – R4 are row replicates. Blots on nitrocellulose membrane were color developed using Fast Red/Naphthol complex as chromogenic substrates.
Molecular studies

RT-PCR detection of PNRSV from apple, peach and apricot

RT-PCR successfully detected PNRSV-viral RNA from apple, peach, and apricot tissues (Fig. 5). A full length CP gene DNA fragment about 704 bp in size was detected from the three tested isolates of PNRSV using specific primers for PNRSV-CP gene (Fig. 5). No signal was detected in the negative control.

DISCUSSION

Characterization of the present isolate of PNRSV was based on symptomatology, chemical and physical properties, serology, molecular analysis and electron microscopy.

Symptomatology

The described symptoms on infected apple trees are similar to symptoms caused by PNRSV infection on several stone fruits (Pusey and Yadava, 1991; Mink, 1992; Abdel-Salam et al. 2008a).

Serologic study

The tested PNRSV isolates from peach, apricot, and apple reacted serologically with a local antiserum prepared for PNRSV-peach isolate. As expected intensity of the reaction was correlated with degree of homology between the antiserum and its respective isolate; being strong with the homologous peach isolate and moderate with the heterologous apricot and apple isolates. Such results agree with results of Crosslin and Mink (1992), Spiegel et al. (1999), and Abdel-Salam et al. (2006a) who reported the serologic diversity between PNRSV isolates.
Virus purification

The purified virus preparations had physical characters typical to nucleoproteins obtained for several isolates of PNRSV (Fulton, 1981; Crosslin and Mink, 1992; Abdel-Salam et al., 2006a, 2008a, b). The EE purification method used in the present study was also successful in purifying other ilarviruses (e.g. PNRSV (Abdel-Salam et al., 2006a, 2008a, b), some begomoviruses (Abdel-Salam, 1999, Abdel-Salam et al. 2006b), an ipomovirus, and a crinivirus (Abdel-Salam, 2012). The EE technique is fast, low cost, and meets the demands of many moderately equipped laboratories.

Electron microscopy examination

Purified virions had an average diameter of 26 nm thus resembling members of Ilarvirus as reported by (Brunt et al, 1996) and Abdel-Salam et al (2006a, 2008a, b).

Molecular studies

Specific primers for the CP gene of PNRSV amplified the full length CP from PNRSV-infected apple, peach and apricot; confirming therefore the presence of PNRSV in the tested isolates. Similar results were obtained by other authors using RT-PCR for the detection of PNRSV in stone fruits (Aparicio et al., 1999; Moury et al., 2001; Ulubas and Ertunc, 2004).

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