Partial characterization of *Necrotic ringspot virus* on apple in EGYPT

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ABSTRACT

A severe isolate of *Necrotic ringspot virus* (NRSV) 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 *Ilarvirus* amplified products similar to those produced from peach and apricot isolates of NRSV-infecting stone fruits. Dot blotting immuno-binding assay (DBIA) showed positive reaction between NRSV-infected apple sap and an Egyptian antiserum for NRSV. 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: *Necrotic ringspot virus*, *Ilarvirus*, RT-PCR, Dot blotting immuno-binding assay, Egypt

INTRODUCTION

NRSV belongs to the family *Bromoviridae*, genus *Ilarvirus* (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 *Ilarvirus* 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).

NRSV 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 NRSV.

NRSV is the most common virus infecting *Prunus* species as peach and apricot (Mink, 1992, Myrata *et al*., 2003; Abdel-Salam *et a*l., 2008a), rosaceous plants (Abdel-salam *et al*., 2008b), and naturally infecting other non-rosaceous plants (Abdel-Salam *et al*., 2006a). NRSV is responsible for yield losses of up to 15% in sweet cherry and up to 100% in peach. NRSV 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 NRSV 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 NRSV is reported on apple (*Malus domestica*). Severe symptoms mimic infections with NRSV 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 NRSV on apple, viz. NRSV-Apple.

MATERIALS AND METHODS

Virus isolates

An isolate of NRSV 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 globsa* as described by Abdel–Salam *et al*. (1997, 2006a). Isolates for NRSV 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*., 2014) was used in measuring virus presence in tested hosts and serologic relationships between NRSV isolates from apple, peach and apricot. Tissue samples were ground, filtered and diluted 1/10 in PBST buffer. An Egyptian antiserum for NRSV 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 NRSV-infected gomphrena (*Gomphrena globosa*) plants, previously inoculated with NRSV-Apple, were used in virus purification. Purification of NRSV-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 NaH_2PO_4-Na_2HPO_4, pH 7.0, containing 1 mM EDTA, 20 mM Na_2SO_3, and 0.1% of each of 2-mercaptoethanol 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 1mM 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 spectrophoto-metrically.

Electron microscopy

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

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Genomic studies

Extraction of total RNA

Total RNA was extracted from *NRSV*-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 CGC 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 NRSV *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^{o}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), *Promegam*, 1 µl dNTPs mix (10mM), 0.25 µl of ribonuclease inhibitor (40 U/ µl), *Promega,* and 8.125 µl DEPC H_2O. The mixture was incubated at 42^{o}C for 1 h, incubated at 95^{o}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 Mg_2Cl, *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 H_2O. PCR conditions included 5 min at 95^{o}C; followed by 35 cycles of amplification of 1 min at 95^{o}C, 1 min at 53^{o}C, 1 min at 72^{o}C, and held for 10 min at 72^{o}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 ringsspot 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 NRSV-Apple isolate. Results in Figure (2) showed the positive reaction of sap from infected apple, apricot and peach with the induced antiserum for NRSV-Peach isolate. The peach isolate of NRSV reacted strongly with its homologous antiserum. While both isolates of apple and apricot of NRSV reacted moderately with the antiserum of NRSV-Peach indicating distant serologic relationship with the peach isolate of NRSV.

Virus purification

The purified virus preparations, using EE technique, for NRSV-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 NRSV).

(e.g NRSV (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 NRSV showed spherical virions with an average diameter is 26 nm (Fig. 4).

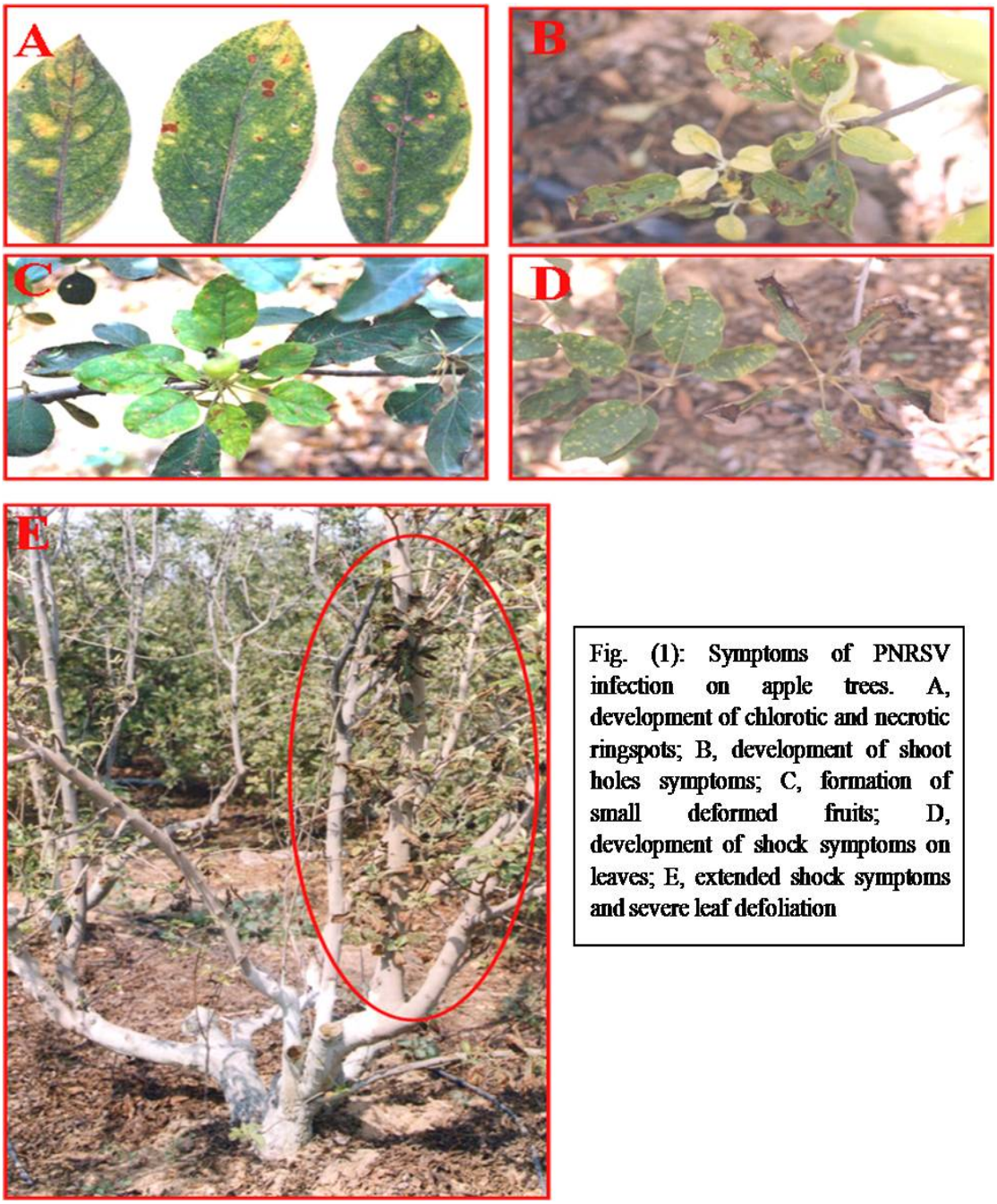

Fig. (1): Symptoms of PNRSV infection on apple trees. A, development of chlorotic and necrotic ringspots; B, development of shoot holes symptoms; C, formation of small deformed fruits; D, development of shock symptoms on leaves; E, extended shock symptoms and severe leaf defoliation

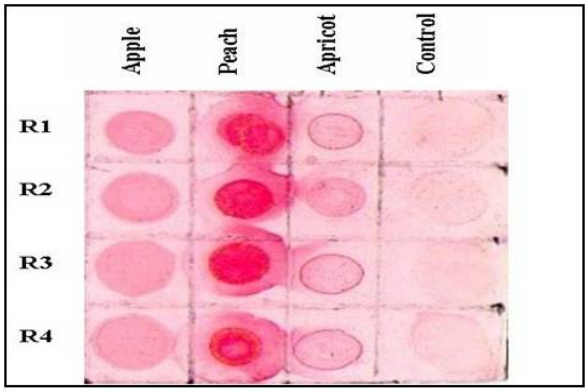

Fig. (2): DBIA showing the detection of PNRSV from infected apple, peach, and apricot trees using PNRSV antiserum prepared for PNRSV-peach isolate (at 1/1000 dilution in PBS buffer) . Control represents healthy sap from apple. R1 – R4 are row replicates. Blots on nitrocellulose membrane were color developed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

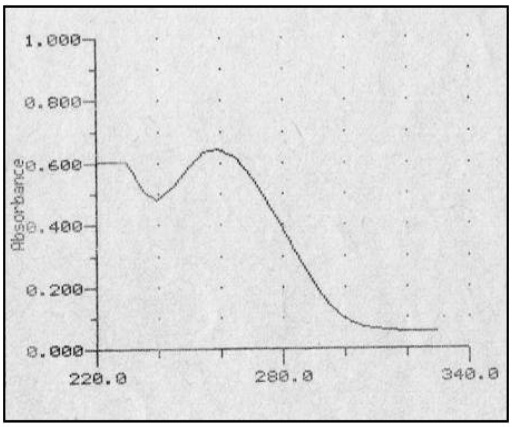

Fig. (3): Ultra-violet spectrum of purified PNRSV-Apple by the method of electro elution (EE).

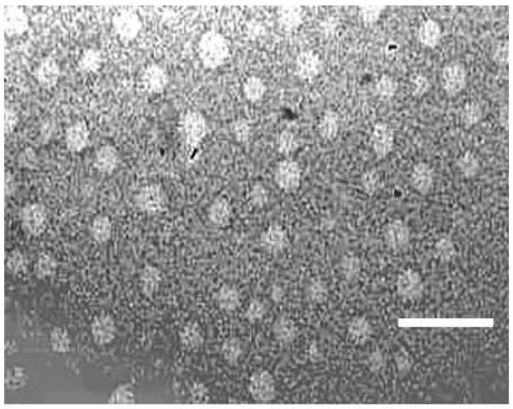

Fig. (4): Electron micrograph showing purified virions of PNRSV purified from infected apple. Bar = 100 nm

Molecular studies

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

RT-PCR successfully detected NRSV-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 NRSV using specific primers for NRSV- CP gene (Fig.5). No signal was detected in the negative control.

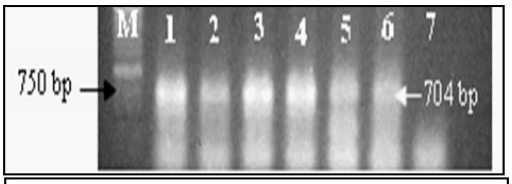

Fig. (5): RT-PCR results showing the amplification of the full length PNRSV-CPs from total RNA extracted from samples obtained from infected apples (1-4), infected peach (5), infected apricot (6). 7, healthy apple.

DISCUSSION

Characterization of the present isolate of NRSV 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 NRSV infection on several stone fruits (Pusey. and Yadava, 1991; Mink, 1992; Abdel-Salam *et al*. 2008a).

Serologic study

The tested NRSV isolates from peach, apricot, and apple reacted serologically with a local antiserum prepared for NRSV-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 NRSV isolates.

Virus purification

The purified virus preparations had physical characters typical to nucleoproteins obtained for several isolates of NRSV (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 NRSV (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 NRSV amplified the full length CP from NRSV-infected apple, peach and apricot; confirming therefore the presence of NRSV in the tested isolates. Similar results were obtained by other authors using RT-PCR for the detection of NRSV in stone fruits (Aparicio *et al*., 1999; Moury *et al*., 2001; Ulubas and Ertunc, 2004).

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