Characterization of two isolates of *Prunus necrotic ringspot virus (PNRSV)* from peach and apricot in Egypt

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ABSTRACT

Two isolates of PNRSV were isolated from peach and apricot trees at the Experimental Station of the Faculty of Agriculture, Cairo University, Giza, Egypt. The peach isolate of PNRSV (PNRSV-PF) was differentiated from the apricot isolate (PNRSV-AP) by ten differential host species. Both isolates were purified successfully with the electro-elution technique. Both isolates had A_{max} and A_{min} at 260 and 240 nm respectively. The A260/280 ratios were 1.55 and 1.60 for the AP and the PF isolates, respectively. Electron microscopy examination showed spherical virions with ca 27-29 nm in diameter. Both isolates had molecular weight of coat protein subunits of 29 kDa, determined by polyacrylamide gel electrophoresis (SDS-PAGE). Antisera raised against the two isolates detected their counter antigens in peach and apricot trees. Both antisera cross reacted with their homologous and heterologous antigens in dot blot immunoassay (DBIA) and agar-double diffusion (ADD) tests. The two virus isolates appeared to belong to the same sero-group and represent two different pathotypes. PNRSV-AP varied in sero-grouping of that of beet necrotic ringspot ilarvirus, a tentative isolate of PNRSV, when examined in ADD test. Indirect ELISA showed that 64.2 % of 210 tested apricot trees were infected, while 150-inspected peach trees showed 29.5 % infection. Four sets of primers were used to amplify both movement protein (MP) and coat protein (CP) genes of the two Egyptian isolates of PNRSV isolated from apricot and peach trees. Amplicons of the correct size (~ 894 bp) for the MP gene and (~ 704 bp) for the CP gene were obtained from the two examined isolates of PNRSV. Nested PCR using specific primers for both the MP and the CP genes confirmed the authenticity of the PCR amplified products. RT-PCR detected successfully the presence of PNRSV in the pollen grains of infected apricot and peach trees. Nucleotide sequences of the MP genes of the two isolates were revised by the GenBank and given the accession # EU100388 for the peach isolate and EU106649 for apricot isolate. Phylogenetic analysis of RNA 3-MP showed ca 65 % similarity between PNRSV-AP and PNRSV-PF; indicating that the two isolates of PNRSV are distantly related. The relatedness between the two isolates and other PNRSV isolates is discussed.

Key words: *PNRSV, Ilarvirus, electron microscopy, DBIA, purification, movement protein gene, RT-PCR, IC-RT-PCR, nested PCR, peach, apricot.*

INTRODUCTION

Prunus necrotic ringspot virus (*PNRSV*), *Bromoviridae*, belongs to the 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 (Crosslin and Mink, 1992; Spiegel *et al.*, 1999), and restriction fragment length polymorphism profiles (Spiegel *et al.*, 1999; Ulubas and Ertunc, 2004). *PNRSV* is graft and pollen-transmitted and potentially seed borne (Uyemoto *et al.*, 1992; Amari *et al.*, 2004). These properties contribute to its rapid spread in stone fruit trees and its worldwide distribution (Marbot *et al.*, 2003). *PNRSV* has been detected in several countries in the Middle East including Syria (Ismaeil *et al.*, 2003), Lebanon (Choueiri *et al.*, 2001), Palestine (Jarrar *et a*l., 2001), Egypt (Ghanem, 2000) and in many other Mediterranean countries (Cyprus, Greece, Italy, Malta, Tunisa, Turkey, Spain) (Myrta *et al.*, 2001).

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).

PNRSV are transmitted by infected root stocks, grafting, and pollen and seeds of apricot and peach. Several *Thrips* spp. have been mentioned to play a role in transmitting the virus (Fulton, 1983; Mink, 1992; Amari *et al.* (2004).

PNRSV causes fruit yield losses (Uyemoto *et al.*, 1992) and affects fruit maturity or tree growth of many commercial *Prunus* spp. (e.g., peach, plum, apricot, sweet cherry, and almond) (Mink, 1992). Consequently, this virus is a component of many phytosanitary certification programs for fruit trees. The detection of *PNRSV* in fruit trees tissues is problematic because virus concentration fluctuates between seasons and the virus is unevenly distributed in infected trees (Scott *et al.*, 1989; Torrance and Dolby, 1984).

PNRSV has recently been observed to cause extensive economic losses to peach (*Prunus persicae* L.) and apricot *(P.armaniaca* L*)* trees in Egypt. Symptoms are mostly expressed as necrotic ringspots on leaves, bud failure and poor quantity and quality of fruits. The present study has been conducted to differentiate between two isolates of *PNRSV* infecting peach and apricot trees in the Giza Governorate, using biological, biochemical, serological, and molecular techniques.

MATERIALS AND METHODS

Isolation and propagation of virus isolates

Two isolates of *PNRSV* were isolated from peach and apricot trees at the Experimental Station of the Faculty of Agriculture, Cairo University, Giza, Egypt. The peach isolate was isolated from peach flowers and was designated as *PNRSV*-PF. The apricot isolate was isolated for apricot leaves and designated as *PNRSV*-AP. Both isolates were purified biologically by mechanical inoculation on *Chenopodium quinoa* and *Gomphrena globsa* as described by Abdel–Salam *et al.* (2006). The beet necrotic ring spot ilarvirus (Abdel-Salam *et al.*, 2006) was used in some experiments as a positive control.

Host rang studies

Different plant species (10 plants, each) were mechanically inoculated with each

isolate and maintained for 45 days in the greenhouse for symptom development. STEP buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 8.3, containing 0.02 M of Na_2SO_3 and ethylene diamine tetracetate (EDTA), and 1.5 % Triton X-100) were used in mechanical inoculation (Abdel-Salam, 1999). Plants were washed thoroughly to evade the corrosive action of the buffer. Non-inoculated plants were left as a control host range.

Physical and chemical studies

Virus purification

Purification of the two virus isolates utilized the electro-elution (EE) technique described by Abdel-Salam (1999) and the sucrose density gradient centrifugation described by Crosslin and Mink (1992).

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 spectrophotometrically.

Electron microscopy

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

SDS- polyacrylamid gele electrophoresis:

Molecular weight analysis for the two virus isolates was preformed according to (Crosslin and Mink, 1992).

Serologic studies

Antiserum production

Antisera for *PNRSV*–PF and *PNRSV*–AP isolates were induced in rabbits as described by Abdel-Salam *et al.* (2006)

Serologic tests

A) Dot blotting immunobinding assay (DBIA):

DBIA test, described by (Abdel-Salam 1999) was used in measuring virus presence in tested hosts and serologic relationships between *BNRSV* and *PNRSV* isolates.

B) Agar double diffusion test (ADDT)

ADDT (Abdel-Salam *et al.*, 2004) was conducted to measure the serologic relationship between the purified *PNRSV* isolates .

C).Indirect enzyme linked-immunosorbent assay (ELISA).

The indirect ELISA method was similar to that described by Converse and Martin (1990) to detect *PNRSV* isolates in peach and apricot orchards. To avoid the problem of the non-equal virus distribution, a compound-sample system was followed where each sample/tree contained four leaves representing the four directions. Samples were collected at the beginning of the Spring season. Samples were collected from the orchards of the Experimental Farm of the Faculty of Agriculture, Cairo University.

Genomic studies

Primer selection

The oligonucleotide primers listed in Table (1) were synthesized (Qiagene Co.) to amplify the coat protein (CP) and movement protein (MP) genes of *PNRSV* (AP & PF) isolates as described in Fig. (1). Oligonucleotide primers used in the RT-PCR were

derived from the published sequences of *PNRSV* RNA-3 (Scott *et al.*, 1998).

The primer set CP (+) sense primer and CP (-) antisense primer complementary to the conserved region of the coat protein gene were used to generate 704 bp fragments from *CP* gene (RNA-3). The nested primers CPNRV (+) and CPNRV (-) were used to amplify 350 bp DNA fragment from the coat protein gene. To amplify the full length of the movement protein gene the primer MP (+) and MP (-) antisense primer were used. The two nested primers (MPNP) and (MPNM) were used to generate 304 bp nested fragment (Table 1).

Extraction of total RNA

Total RNA was extracted from *PNRSV*-infected peach and apricot plants by applying the technique of high pure RNA Tissue kit (Roche Molecular Biochemicals, (cat. No 2033674).

RT-PCR

First strand cDNA was synthesized in a 20 µl reaction containing 2 µl of total RNA (~10 µg), 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 1 mM each of dNTP, 40 units of ribonuclease inhibitor (Amersham International, Cleveland), 2 units of AMV-RT enzyme (Roche), and 1 µg of each antisense primer separately.

The RT reactions were carried out with AMV reverse transcriptase (Roche) and subsequent PCR amplification was done by using *Taq* DNA polymerase (Roche). PCR cocktail included: 2.5 µl of the reverse transcription products, 2.5 µl of 10x buffer (166 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl at pH 8.8, and 0.1% Tween 20), 2 mM $MgCl_2$, 0.2 mM each of dNTP, and 25 pmol of each primer in a total volume of 25 µl. The cDNAs were amplified for 35 cycles of 94 °C for 1 min, 52-65 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. All PCR reactions have been optimized using a technique developed by Cobb and Clarkson (1994) using different annealing temperature (TA) (52, 55, 60, and 65 °C) according to the gene amplified (Table 1), concentration of cDNAs, $MgCl_2$, dNTPs and primers in order to obtain the best amplification of PCR products. The RT-PCR fragments were analyzed on 1 % agarose at 60 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide.

Molecular cloning

The DNA fragment (894 bp) encoding the full length sequence of the *MP* gene of *PNRSV*/ (AP & PF isolates) was purified from the agarose gel using GFX Agarose Gel Extraction Kit (Amersham, Pharmacia, Uppsala.) and ligated into the pGEM-T Easy plasmid vector system supported by Promega (Madison, Wisconsin, USA) according to Maniatis *et al.* (1987) and Spiegel *et al.* (1999). The protocol of Hanahan (Hanahan and Meselson, 1983) was used for bacterial transformation in *E. coli* (JM109) cells with the ligation reactions. The recombinant DNA plasmids containing the *MP* gene of *PNRSV* was isolated from transformed cells using the High Pure plasmid preparation kit (Roche). Successful clones were validated by PCR using MP (+) / MP (-) primers.

Sequence analysis

PNRSV/MP (Peach & Apricot isolates) DNA fragments were sequenced by ABI-PRISM™ 3100 Genetic Analyzer by using dye-primer and dye terminator method at Gene Link DNA Sequencing service, NewYork, USA. The resulting sequence for *MP* gene was then compared to published sequences of *PNRSV* isolates (Table 2) using the Clustal W method (Thompson *et al.*, 1994).

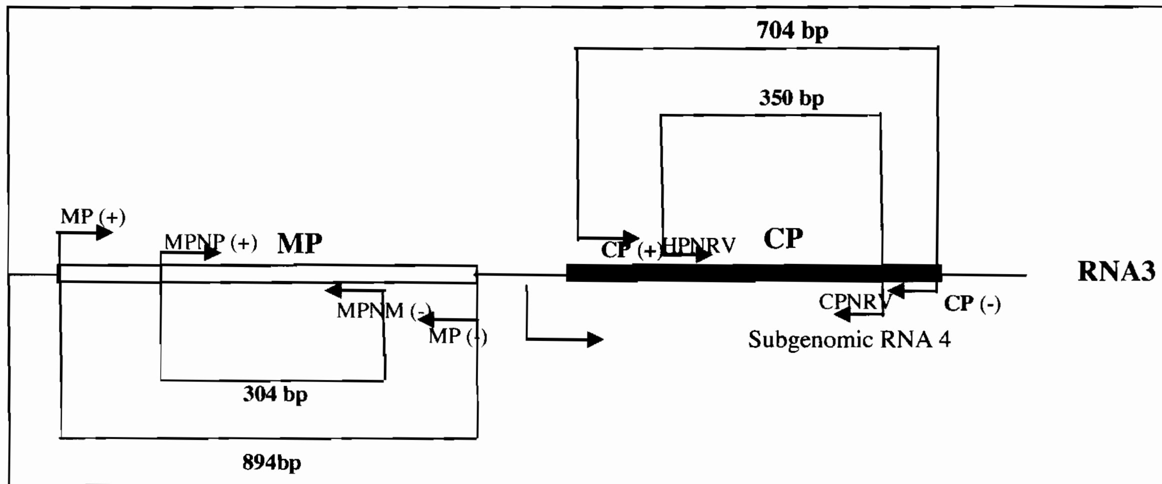


Fig. (1): Schematic diagram of Prunus necrotic ringspot virus (PNRSV) RNA3 showing the location of primers used for RT-PCR amplification and for cloning and sequencing of the two isolates of PNRSV isolated from Apricot and Peach.

Table (1): Primers used in the RT-PCR amplification of PNRSV coat protein (CP) and Movement protein (MP) genes.

Primer I.D.	Sequence 5' ⟶ 3'	Nucleotide position	Gene name	Size of PCR product	References
MP (+)	GTT GGT TGA ATA GTG TTT CAG TAT GGC C	158-186	Full length MP gene	894 bp	Scott *et al* (1998)
MP (-)	CGC TCG TGA CAT AGT CAC	1034-1052			
MPNP	AGA CGT CGT GAC AGA CGT CGA AG	537-559	Nested for MP gene	304 bp	
MPNM	TTC TGT ACC TGC CAA TAT CCT ACT TCG	814-841			
CP (+)	CCG AAT TTG CAA TCA TAC CCA CGC T	1116-1140	Full length CP gene	704 bp	
CP (-)	CGG AGA AAT TCG AGT GTG C	1800-1820			
CPNRV (+)	CGA CCG CAA CCG GTC GTC AAG ACC A	1277-1302	Nested for CP gene	350 bp	
CPNRV (-)	TCT TGA ATT CGA CAC GAT G	1648- 1667			

Table (2): Foreign isolates of PNRSV used in phylogenic studies in this paper. GenBank accession numbers are indicated in parentheses.

PNRSV isolates	Original host	Geographic origin	References
P8450	Peach, May Crest	Italy (AJ306815)	Aparicio and Pallas (2002)
P4090	Peach, Marylin	Italy (AJ306817)	Aparicio and Pallas (2002)
Valticka	Peach	Czech Republic (AF170171)	Vaskova *et al.* (2000)
Prunus persica	Peach leaves	India (AM712615)	Chandel *et al.* 2007
A8360	Apricot, Tirynthos	Italy (AJ306812)	Aparicio and Pallas (2002)
A9170	Apricot, Napoletana	Italy (AJ306811)	Aparicio and Pallas (2002)
A3430	Apricot, Cafona	Italy (AJ306810)	Aparicio and Pallas (2002)

RESULTS AND DISCUSSION

Field symptoms of *PNRSV* on peach and apricot

Infected peach and apricot trees showed prominent symptoms of bud failure. Necrotic ringspots and shot holes were very obvious on infected leaves (Fig.2). Diseased trees showed bark splitting and reduction in trunk circumference. These observed symptoms are typical to the symptoms caused by *PNRSV* (Fulton, 1981; Uyemoto, 1992; Pusey and Yadava, 1991) Fruit setting is more dramatically reduced in apricot tree than in peach due to cessation of development of flowers and flower dropping. Similarly, Pusey and Yadava, (1991) and Uyemoto (1992) have measured a reduction in peach yield reaching (8.2-47.3%) and 30%, respectively according to grown variety. Amari *et al.* (2004) have shown that *PNRSV*-infected pollen of apricot had a germination rate of only 27%, while healthy pollen shows 64%. This latter observation can account for the undeveloped flowering in apricot due to failure of pollen germination and hence failure of female gamete fertilization.

Host Rang Studies

Results in Table (3) indicated that only ten plant species of the tested hosts were able to differentiate between the two virus isolates. These species included *Lactuca sativa L.*, *Xanthium pungens* L. (*Compositae*) , *Cucumis pubescens* L, *Cucumis sativus* L. cvs. Beit Alpha, Hybrid 9, and Hybrid 14 (*Cucurbitaceae*), *Vicia faba* L., *Vigna Unguiculata* Walp. (*Leguminosae*),*N. tabacum* L. cvs. White Burley and Java, *N. benthamiane* L., and *S. nigrum* L. (*Solanaceae*). Reactions of peach or apricot to PF and AP isolates were similar. Reactions of *C. quinoa* Willd and *Gomphrena globosa* L. to PF and AP virus isolates were typical to the known reactions of *PNRSV* isolates to these hosts described by Fulton (1957). However, the tested cultivars of *Cucumis sativus* varied in their reactions to *PNRSV* isolates; an indication of the viabilities between *PNRSV* isolates in pathogenecity as reported by (Howell and Mink, 1988).

Physical and chemical studies

Virus purification

Purified *PNRSV*-AP isolate migrated in the sucrose gradients forming three components as expected. The top components showed no nucleoprotein pattern. Whereas, the middle and bottom components showed A260/280 values between 1.2 and 1.1 for the middle and the bottom fractions, respectively (Fig. 3A); an indication of capsid protein destabilization as known for most ilarviruses (Fulton, 1981).

The absorbance values of the purified *PNRSV*-PF and AP applying the EE technique

(Table, 4 and Fig. 3B) were typical of that described by Fulton (1981) and Crosslin and Mink (1992) for *PNRSV*.

The above results showed the benefits of the EE in maintaining the integrity of the purified virus comparing to the sucrose gradient method.

Electron microscopy

Purified virus preparation of AP and PF, obtained from the EE method, showed virions with *ca* 27-29 nm. Few virions were 23 nm in diameter (Fig. 4-A&B). The variability in virion diameters probably reflects the nature of *PNRSV* as being labile to negative staining which probably induces various degree of virion swelling upon staining. The obtained diameters of purified virions agree with the reported diameters for *PNRSV* virions by Kurihara *et al.* (1998), Brunt *et al.* (1996), and Ghanem (2000).

SDS- polyacrylamid gele electrophoresis

Molecular weight of coat protein subunits for both *PNRSV*-AP and PF was *ca.*29 kDa for both virus isolates (Fig.5). Such a value is within the range reported for *PNRSV* by Crosslin and Mink (1992).

Fig. (2): Observed symptoms of PNRSV*-PF *infection on peach and PNRSV*-AP *on apricot trees showing bud failure in peach (A) and apricot (B) and necrotic ringspots and shot holes on leaves of peach (C) and apricot (D)

Table (3): Host range of Prunus necrotic ring spot virus (AP, PF isolates) tested by mechanical inoculation.

Tested Plants	Common name	AP	PF
Amarnthaceae			
Gomphrena globosa L.	Globe amaranthus	ChLL, NLL	ChLL, NLL
Chenopodiaceae			
Beta vulgaris L			
Athospoly		NT	Mot
cv. Florida		-	NT
cv. Kawmera	Sugar beet	VC	NT
cv. Oscar Poly		E	NT
cv. Pamela		E, Mot	NT
Beta patelaris L.	Table beet	-	-
C. amaranticcolor Coste & Reyn	Goss foot	NLL	NLL
Chenopodium murale	Goss foot	-	-
C. quinoa Willd	Goss foot	ChLL, NLL, Mot	ChLL, NLL, Mot
Spinacea oleracea L.	Spinach	-	-
Compositae			
Lactuca sativa L.	Lettuce	NRS.	-
Hilianthus annuus L.	Sun flower	-	-
Xanthium pungens L.	Spring cocklebur	M, NRS.	-
Zinnia elegans Jacq	Golden zinnia	-	NT
Cucurbitaceae			
Citrullus vulgarris L.	Water melon	Mot	Mot
Cucumis melo L.	Melon	M	Mot
Cucurbita pepo L.	squash	Mot	Mot
Cucumis pubescens L.	Hairy cucumber	L	-
Cucumis sativus L. cvs.			
Beit Alpha		VC,LP	ChLL, NLL
Hybrid 9	Cucumber	VC,LP	Mot
Hybrid 14		VC,LP	Mot
Euphorbiaceae			
Ricinus communis	Castor Bean	-	NT
Leguminosae			
Arachis hypogaea L.	Pea nut	-	-
Cyamopsis tetragonoloba (L.) Taubert	Guar,	ChLL, NLL	ChLL, NLL
Glycine max L.	Soybean	-	-
Lucaenia alba		LP	NT
Phaseolus vulgaris L.*Giza 6*	Green bean	-	-
Pisum sativum L.	pea	VY.	NT
Vicia faba L.	Broad bean	L.P.	-
Vigna Unguiculata Walp.	Cowpea	L.P.	-
Rosaceae			
Prunus armaniaca L.	Apricot	ChRS,NRS,SH,BF	ChRS,NRS,SH,BF
Prunus persicae L.	Peach	ChRS,NRS,SH,BF	ChRS,NRS,SH,BF
Solanaceae			
Datura stramonium L.	Datura	-	-
Datura metal L.	Datura	-	-
Nicotiana glutinosa L.	Tobacco	-	-
N. tabacum L. cv. *White Burley*	Tobacco	Mot	-
N. tabacum L. cv.*Java*	Tobacco	Mot	-
N. benthamiane L.	Tobacco	M	Mot
N. glutinosa L.		-	-
Solanum melanogena L	Egg plant	-	-
S. nigrum L.	Black Nightshade	Mot	-

BF = Bud failure; ChLL = Chlorotic local lesions; E = Enation; NLL = Necrotic local lesions; NT = Not tested; ChRS. = Chlorotic ring spot; L = latent symptoms; LP = Line pattern; MD = marginal deformation; NRS = Necrotic Ring spot; S = Stunting; SH = Shot holes; VB = Vein banding; VC = Vein clearing; VY. = Vein yellowing; M= mosaic; Mot = mottling; NLL = necrotic local lesion; All inoculated plants were tested with their corresponding antisera using DBIA; - = Negative for DBIA.

Table(4): Ultra-violet absorbance values of purified PNRSV-AP and PF isolates using the electro elution technique.

Measured Values	ABSORBANCE (A)	
	PNRSV-AP	*PNRSV*-PF
A max	260 nm	260 nm
A min	240 nm	240 nm
A 260 nm	0.418	0.600
A 280 nm	0.307	0.315
A 260/280 ratio	1.55	1.60
Virus yield (mg/g tissue)	0.69	0.87

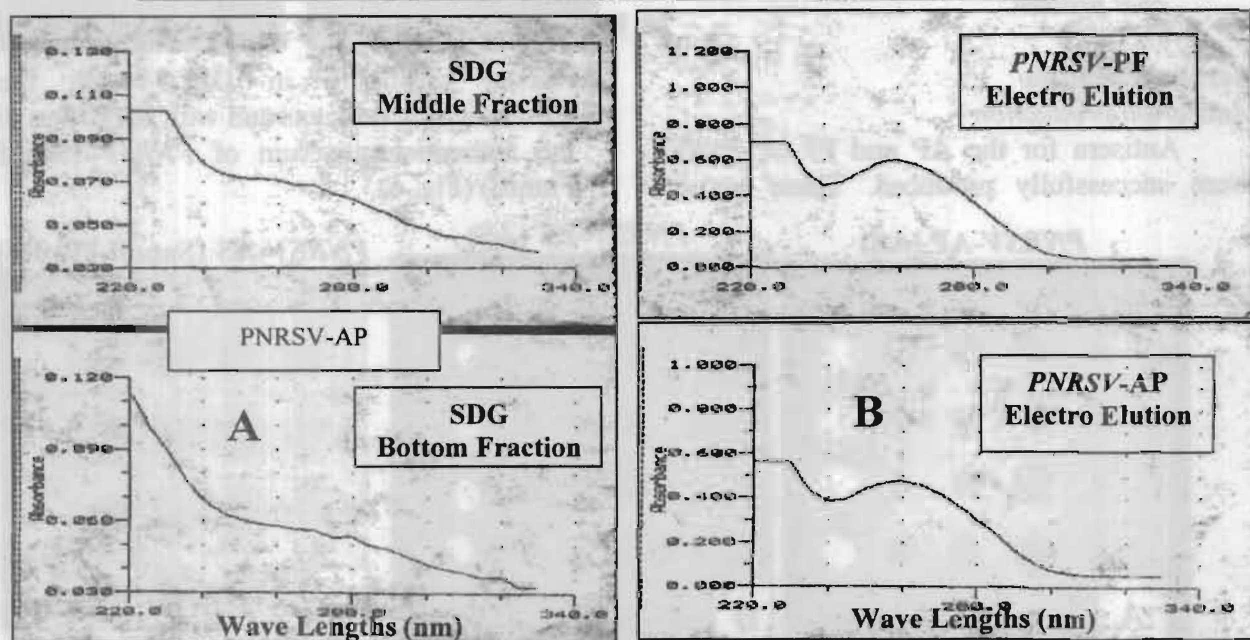


Fig. (3): Ultraviolet spectra of (A) middle and bottom fractions recovered from purified PNRSV-AP by sucrose density gradient centrifugation; (B), purified PNRSV-PF and PNRSV-AP by the method of electro elution (EE).

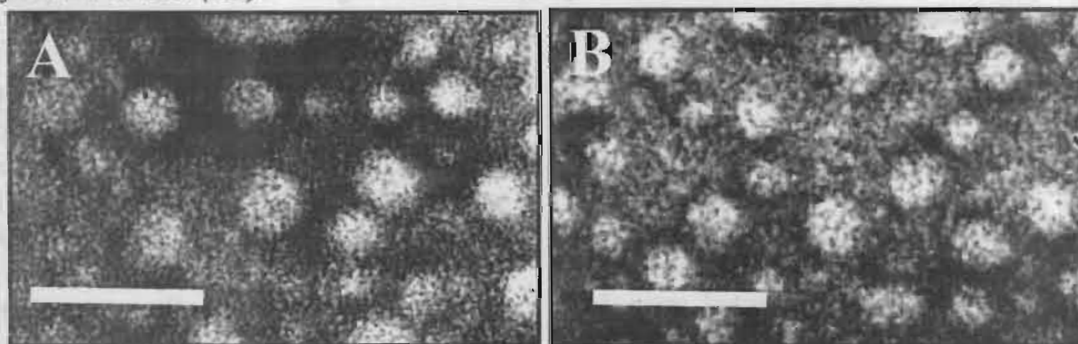


Fig. (4): Electron micrographs showing the purified PNRSV virions recovered from the electro elution method. (A), PNRSV-AP; (B), PNRSV-PF. Bar = 100 nm.

Fig. (5): A, SDS-PAGE showing the migration of coat protein subunits of PNRSV-AP and PNRSV-PF recovered from sucrose density gradients (SDG) and electro elution (EE) methods. B, as in A but with further purification of the 29 K fraction through EE. Arrows refer to the position of the 29 K coat protein.

Serologic studies

Antiserum production

Antisera for the AP and PF of *PNRSV* were successfully produced. These antisera reacted positively in DBIA with their corresponding antigens and with *BNRSV* as did the authentic antiserum of *PNRSV* (Sanofi-France) (Fig. 6).

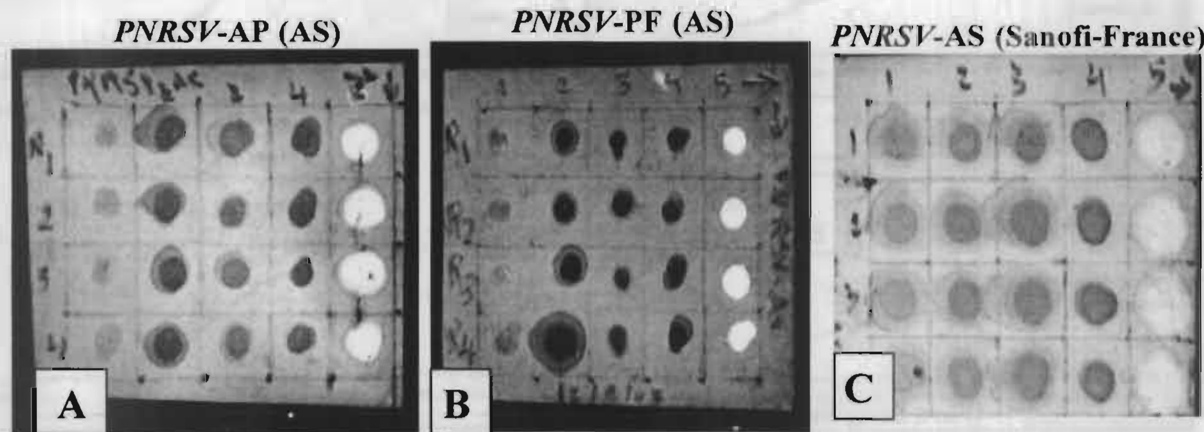


Fig. (6): DBIA showing the reactions of A) PNRSV-AP(AS), B) PNRSV-PF(AS) and PNRSV(AS) Sanofi-France wit purified preparations of : Columns 1, 1.0 mg/ml PNRSV-AP; 2, 2.1 mg/ml PNRSV-AP; 3, 3.4 mg/ml PNRSV-PI 4, 2.0 mg/ml BNRSV; 5, healthy preparations. Antisera were diluted 1/1000 with TBST buffer, pH 8.(Naphthol/Fast red complex were used as chromogenic substrate. Antigens were blotted as 1 µl/square. Each ro was repeated 4 times (R1-R4).

Serologic relationship between PNRSV isolates :

A) Dot blotting immunobinding assay (DBIA)

Results of DBIA test (Fig. 7) showed that *BNRSV*-As was more permissive than both *PNRSV* antisera. *BNRSV*-AS reacted strongly with its homologous antigen and moderately (i.e., gomphrena, apricot and apple) to poorly with the other antigens (i.e., pear and peach). *PNRSV*-AP and PF were similar in their serologic reaction with their corresponding antigens and other tested anitgens. However, they give poor reaction with *BNRSV* antigen. These results indicate the specificity of the tested antisera with their corresponding antigens.

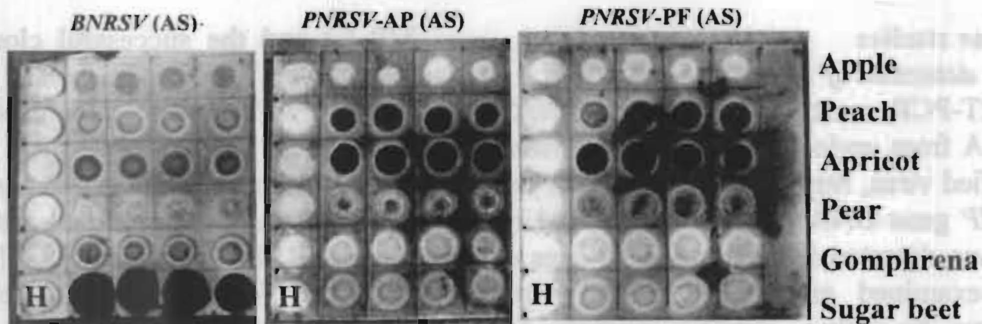


Fig. (7): DBIA test showing the serologic relationships between BNRSV(AS), PNRSV-AP(AS), and PNRSV-PF(AS) upon reaction with screened filed samples from apple, peach, and apricot and , gomphrena, (G. globosa) and sugarbeet (Beta vulgaris) plants collected from the greenhouse and inoculated with either of BNRSV, PNRSV-AP, or PNRSV-PF. Samples were ground (1:4, w/v) in 0.01 M STEP buffer. Primary antisera were diluted 1/1000 with TBST, while secondary antiserum was diluted 1/2000. Naphthol/Fast Red were used as chromogenic substrates.

B)Agar double diffusion test (ADDT)

All the *PNRSV*-AP and PF isolates, and the presumptive *BNRSV* isolate of *PNRSV* reacted with the AP and PF antisera (Fig. 8). All tested antigens gave homologous reactions with *PNRSV*-AP antiserum. Both *PNRSV*-AP and PF cross reacted with their corresponding antisera; an indication of their being located in the same serogroup. However, spur formation (see white arrow) was developed between *BNRSV* (C) and *PNRSV*-AP (D) in the presence of *PNRSV*-PF (AS). Such result indicates that *PNRSV*-PF (AS) contains additional antibodies that can differentiate between *BNRSV* and *PNRSV*-AP and both antigens are belonging to different sero-groups of *PNRSV*. *PNRSV* is known to contain different serogroups (Mink *et al*., 1987; Crosslin and Mink, 1992; Spiegel *et al*., 1999).

Detection of PNRSV in the Apricot and Peach orchards by Indirect-ELISA

ELISA data indicated that out of 156 inspected peach trees for *PNRSV*-PF, 46 trees were infected with 29.48% infection. As for apricot, out of 210 tested apricot trees, 135 were positive for *PNRSV*-AP antiserum giving 64.28% infection. These results may indicate the high virulence of the *PNRSV*-AP when compared with the *PNRSV*-PF on peach. Unfortunately, no further study using DAS-ELISA were conducted to examine the pathogenecity of each virus isolate of peach or apricot in the field.

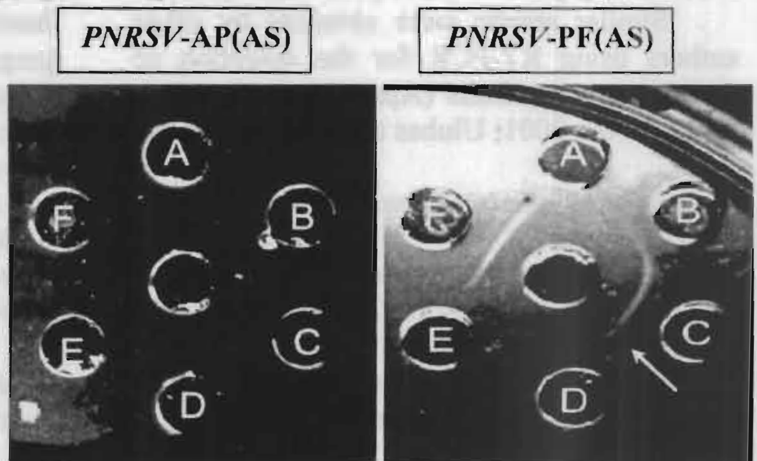


Fig. (8): Agar double diffusion test showing the reaction of PNRSV antisera with different isolates of PNRSV. A& D=PNRSV-AP; B&F=PNRSV-PF; C=BNRSV; E=healthy preparation. Each of the peripheral wells was filled with 50 µl of 1mg/ml purified virus. Central wells were loaded with either PNRSV-AP(AS) or PNRSV-PF(AS) diluted at ¼ with PBS buffer.

Molecular studies

RT-PCR detection of PNRSV/AP &PF :

RT-PCR successfully detected *PNRS*-viral RNA from apricot tissues, peach flowers and purified virus, respectively (Fig.9). A full length *MP* gene DNA fragment about 894 bp in size was detected from the two isolates of *PNRSV* examined and from purified virus preparation by using specific primers for *PNRSV* MP gene (Fig.9). Nested PCR product of MP gene (300 bp) was also amplified from the first PCR amplified product of the Mp gene to confirm the authenticity of the resulting PCR product (894 bp) (Fig.9). Figure (10) shows the RT-PCR amplified fragments (704 bp) full length coat protein gene (CP) RNA3 from apricot and peach flowers (lanes 1&2) and the nested PCR product ~350 bp from the same tissues (lanes 3&4). The amplified DNA fragments are in agreement with the expected size calculated from the positions of the primers and also with the published nucleotide sequence of *PNRSV* (Scott *et al.*, 1998). No signal was detected in the negative control. RT-PCR was also successful in the detection of both virus isolates in the pollen grains obtained from infected peach and apricot trees (Fig. 11). The latter result indicates the potential risk of the pollen borne-*PNRSV* in spreading out the necrotic ringspot disease in peach and apricot.

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).

Molecular cloning and Sequencing of PNRS/AP and PF :

To validate the ligation and cloning of the MP genes of *PNRSV/AP and PF* into pGEM-T-Easy cloning vector, PCR amplification was performed on the recombinant plasmids using the primer set MP (+) / MP (-) and the successful clones containing inserts with expected size (894 bp) of the full length MP were identified by DNA sequencing.

Nucleotide sequence analysis for the MP genes of the two virus isolates was obtained using *ABI*-DNA sequencer. The sequences submitted for analysis and revision by the GenBank were donated the accession # EU100388 for the peach isolate and EU106649 for apricot isolate.

Multiple alignments of the nucleotide sequences of the two isolates under study showed 64.8% similarity with each other and > 73% similarity with some of the Italian isolates (Table 5 and Fig. 12). Phylogenic data seem also to suggest that the two isolates under study are far distantly related to the Valticka isolate (AF170171) (*ca* 44 %); again an evidence of the presence of true larger variations between isolates of *PNRSV*. Studies on the RNA 3 of *PNRSV* by Scott *et al.* (1998) showed, however, high degrees of conservation between *PNRSV* isolates. However, when RNA 4, a subgenomic of RNA 3 was taken into consideration, *PNRSV* isolates were divided into three different groups (Vaskova *et al.*, 2000). Vaskova *et al* (2000) have also referred to the effect of geographical location on diversities between *PNRSV* isolates. From the above discussion, one may deduct that there is diversity between *PNRSV* isolates and geographic locations may play a role in such divergence. In addition, most of the phylogenic studies on *PNRSV* were on RNA3 (Moury *et al.*, 2001) which represents a small part of large genome of 8.056 kb (Brunt *et al.*, 1996); an indication of the need of further future studies on other parts of *PNRSV* genome.

Such results indicate that the AP and PF isolates are distantly related but are still isolates of *PNRSV*. The two isolates have

resemblance in symptomatology on peach and apricot, are grouped altogether in one serologic group, having typical ultraviolet spectra, having both the same molecular weight for the coat protein subunits and similarity in particle morphology; however, they are two different pathotypes. Such variation in sequence homology may some times arise by possible genomic mixing between alien ilarviruses or related non-ilarvirues (e.g. alfalfa mosaic virus, AMV) and the present isolates of ilarviruses. For example, beet necrotic ringspot virus, a new ilarvirus infecting sugar beet, induces symptoms typical to those caused by *PNRSV* on peach and apricot upon artificial inoculation on these two species (Abdel-Salam *et al.*, 2006). Such incidence exemplifies the possible increasing of degrees of risk of genomic mixing in nature. This may alter the properties of the present isolates and may lead to new isolates, different in host range, or even new strains. In fact, Aparicio *et al.* (2001) presented *in vitro* evidence on the recognition of *cis*-acting sequences in the RNA 3 of *PNRSV* by the replicase of AMV which led to the replication of *PNRSV* in a non-host plant. In spite this variation of the MP between the AP and PF isolates, the two isolates show large regions of sequences occur in common. This occurrence may support the idea that the two isolates of *PNRSV* have evolved from a common ancestor and would give an explanation for the strong serological relationships reported between the two isolates in this paper.

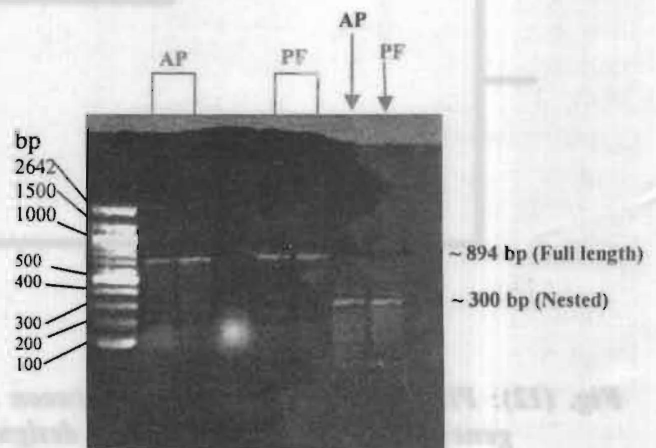


Fig. (9): A multiple RT-PCR result where the 894 bp fragment corresponds to the full length movement protein gene of PNRSV (lane 1& 2 from Apricot tissue and purified virus; Lane 4 & 5 from Peach flowers and purified virus respectively) and the 300 bp fragment corresponds to the nested PCR product amplified from the full length MP gene, i.e. amplified from the first PCR product of the Movement protein gene (Lane 6 (AP) apricot, Lane 7 (PF) peach flowers). Lane 3 & 8: negative control for PCR (No cDNA template). M: Molecular weight DNA marker (100 bp ladder).

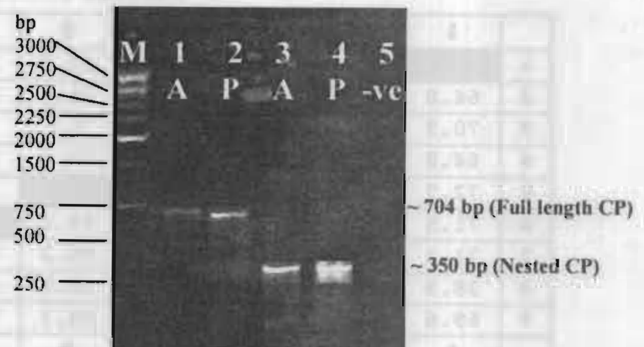
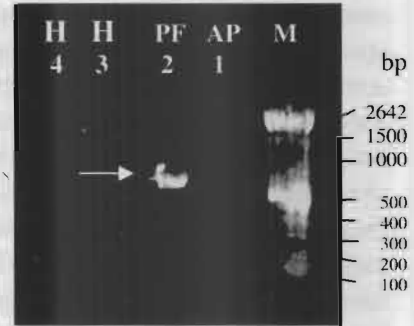


Fig. (10): 1% agarose gel electrophoresis showing the RT-PCR results of PNRSV coat protein gene amplified from total RNA extracted from Apricot (A) and Peach (P) infected tissues where the 704 bp fragment corresponds to the full length coat protein gene of PNRSV (lane 1& 2) and 350 bp fragment corresponds to the nested PCR product amplified from the full length CP gene, (Lane 3 and 4). Lane 5: negative control for PCR (No cDNA template). M: Molecular weight DNA marker (XVI, Roche Applied Science).

Fig. (11): Agarose gel electrophoresis showing the migration of PCR products of PNRSV/CP amplified from pollen grains of apricot (AP) and peach flowers (PF). The total RNAs extracted from AP & PF pollen grains were amplified using the coat protein gene primers CP (+) /CP (-) generating 704 bp (Lanes 1 & 2). Healthy controls (lanes 3 &4). M: 100 bp ladder.



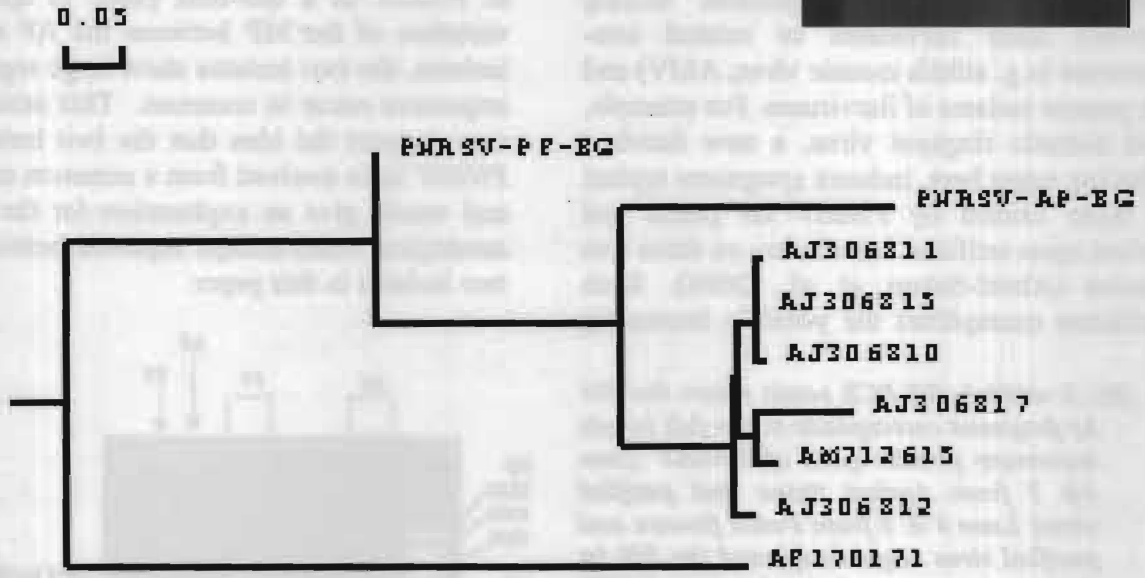


Fig. (12): Phylogenetic relationships between PNRSV-PF and PNRSV/AP movement protein genes with other PNRSV isolates designated with their accession numbers retrieved from the GenBank.

Table (5): Percentages of identity and divergence between PNRSV-AP-EG (Accession # U106649) and PNRSV/PF-EG (Accession # EU100388) movement protein genes with other PNRSV isolates.

Percent Divergence

	1	2	3	4	5	6	7	8	9		
1		35.2	29.1	35.2	27.3	28.5	28.9	61.1	30.4	1	EU106649
2	64.8		27.2	31.6	26.6	27.4	28.0	51.0	28.4	2	EU100388
3	70.9	72.8		11.3	5.1	1.3	1.2	٦.٥٩	5.9	3	AJ306815
4	64.8	68.4	88.7		11.6	11.8	11.5	٠.٦٠	9.9	4	AJ306817
5	72.7	73.4	94.9	88.4		5.4	4.8	٢.٦٠	5.3	5	AJ306812
6	71.5	72.6	98.7	88.2	94.6		1.6	٨.٥٩	6.2	6	AJ306810
7	71.1	72.0	98.8	88.5	95.2	98.4		٤.٦٠	5.5	7	AJ306811
8	38.9	49.0	40.4	40.0	39.8	40.2	39.6		60.3	8	AF170171
9	69.6	71.6	94.1	90.1	94.7	93.8	94.5	39.7		9	AM712615
	1	2	3	4	5	6	7	8	9		

Percent Identity

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الملخص العربي

توصيف عزلتين لفيروس البقع الحلقية الميتة فى البرقوق والمعزولتين من الخوخ والمشمش فى مصر

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تم الحصول على عزلتين من فيروس البقع الحلقية الميتة فى البرقوق prunus necrotic ringspot virus (PNRSV) من اشجار الخوخ والمشمش بمحطة التجارب الزراعية لجامعة القاهرة. وقد امكن التفرقة بين العزلة الفيروسية من الخوخ PNRSV-PF والاخرى من المشمش PNRSV-AP بواسطة عشرة عوائل نباتية مميزة. وقد امكن تنقية العزلتين الفيروسيتين بواسطة تقنية الازاحة الكهربية electro-elution . وبينت نتائج طيف الامتصاص أن أعلى وادنى امتصاص لهما كان عند ٢٦٠ و ٢٤٠ نانوميتر على التوالى. وكانت نسبة الامتصاص للأطوال الموجية A260/280 تبلغ ١,٥٥ و ١,٦٠ لعزلتى المشمش والخوخ الفيروسيتين على التوالى. وأظهرت اختبارات الفحص بالمجهر الالكترونى ان الجسيمات الفيروسية لكلا العزلتين مستديرة وذات قطر يبلغ حوالى ٢٧-٢٩ نانوميتر. وبينت دراسات الهجرة الكهربية باستخدات تقنية SDS-PAGE ان الوزن الجزيئى لوحدات البروتين البنائية يبلغ ٢٩ كيلو دالتون لكلا الفيروسين. وقد نجحت التجارب التى اجريت بغرض الحصول على امصال للعزلتين الفيروسيتين. وكان المصلان المنتجان ذوى فعالية عالية فى التفاعل مع الانتيجينات المقارنة لكلا الفيروسين فى اختبارات dot blot immunoassay (DBIA) and agar-double diffusion (ADD) tests . وهذا يبين انهما تتبعان مجموعة سيرولوجية واحدة وان كانتا متباعدتين من الناحية الممرضة. كذلك بينت اختبارات الـ ADD ان العزلة PNRSV-AP متباينة سيرولوجيا مع فيروس beet necrotic ringspot والمفترض كونه عزلة فيروسية مختلفة لـ PNRSV . وامكن بواسطة اختبارات الـ Indirect ELISA فى الكشف عن نسبة اصابة ٦٤,٢ % بالعزلة PNRSV-AP عند فحص ٢١٠ شجرة مشمش و نسبة اصابة ٢٩,٥ % بالعزلة PNRSV-PF عند فحص ١٥٠ شجرة خوخ. وقد استخدمت فى اختبارات تفاعل البلمرة المتسلسل، بأستخدام انزيم النسخ العكسى reverse transcriptase polymerase chain reaction (RT-PCR)، اربعة ازواج من البادئات للحصول على اجزاء مبلمرة amplicons لجين الـ movement protein (MP) تبلغ 894 bp وجين الـ coat protein (CP) تبلغ 704 bp لكلا العزلتين الفيروسيتين. ولقد استخدمت تقنية الـ Nested PCR مع بادئات خاصة لكل من الـ MP و الـ CP genes لتأكيد النتائج المتحصل عليها. ولقد استخدم بنجاح اختبار الـ RT-PCR لتأكيد تواجد الفيروس فى حبوب اللقاح المأخوذة من اشجار مشمش وخوخ مصابة بـ PNRSV . ولقد تم الحصول على التتابع النوتيدى لجين الـ MP لكلا العزلتين. وتمت مراجعة هذا التتابع بواسطة الـ GenBank والذى اعطى accession # EU100388 لعزلة الخوخ و accession # EU106649 لعزلة المشمش. اظهرت دراسات التتابع النوتيدى على RNA 3-MP للعزلتين الفيروسيتين وجود درجة تشابه تبلغ ٦٥ % تقريبا مما يظهر ان العزلتين ذوات درجة قرابة متباعدة. ولقد قورن هذا التتابع ايضا مع عزلات للـ PNRSV. مسجلة فى بنك الجينات الامريكى.