Beet necrotic ringspot virus, a new ilarvirus infecting sugar beet in Egypt: Biological, biochemical, serological, and genomic studies

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Aly M. Abdel-Salam*, Manal A. El-Shazly, Hayam S. AbdelKader***
*Plant Pathology Department, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt
**Virus Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza 12619, Egypt

ABSTRACT

A new ilarvirus infecting sugar beet plants was isolated for the first time in Egypt. The virus was given the provisional name "beet necrotic ringspot virus-Egypt, BNRSV". BNRSV causes ring spots, line pattern and calico mosaic on sugar beet in the field. It infected several hosts belonging to seven families. BNRSV was transmitted mechanically and through Thrips tabaci insects, but not through Myzus persicae aphids or through the tested seeds of some sugar beet cultivars. Purified BNRSV migrated in sucrose gradients forming three components with an average $A_{260/280}$ of 1.5 and 1.63 for un-fractionated and fractionated components, respectively. Coat protein subunits of BNRSV had a value of 29 kDa. Genomic RNAs were separated into four species of RNAs in agarose gel electrophoresis. Several serologic tests indicated its close ties with the ilarvirus, prunus necrotic ringspot virus, PNRSV. Genomic studies showed the ability of the universal JC10/ JC12 primer pairs for PNRSV to amplify 641 bp of the coat protein gene of both PNRSV and BNRSV. Further, BNRSV coat-protein gene had an Mse I restriction site similar to the severe isolates of PNRSV. Nucleotide sequence analysis of the 641 bp amplified-coat protein gene of BNRSV indicated 65.80 % similarities with the published sequences of ilarviruses. Sequence relationship between BNRSV-EG isolate and PNRSV CH3, CH19, ring 21, and ring 28 isolates was discussed

***Key words**: BNRSV-EG, ilarvirus, sugar beet, genome.*

INTRODUCTION

Prunus necrotic ringspot virus (*PNRSV*), *prune dwarf virus* (*PDV*), and *apple mosaic virus* (*APV*) = *rose mosaic virus* (*RMV*), are positive sense RNA plant viruses with tripartite genomes that belong to the *Ilarvirus* genus (Fulton, 1983, Sanchez-Navarro *et al.*, 1998). The genus *Ilarvirus* refers to isometric labile ringspot viruses (Fulton, 1968). They induce a variety of symptoms in stone fruits from none to a severe symptomatic disease, depending on the isolates (Howell and Mink, 1988; Vaskova *et al.*, 2000). Ilarviruses have their genetic information in their three heaviest RNA species (in a descending order of molecular weight, RNA1, 2, and 3). A fourth RNA species (RNA 4, the coat protein) is sub-genomic of RNA3. These four species of RNA are encapsulated into quasi-isometric

particles of various sizes ranging from 20- 38 nm. *PNRSV* is one of the most important ilarviruses because of its quarantine significance in many countries. It sediments in sucrose density gradients (SDG) forming three components (Gonssalves and Fulton, 1977; Crosslin and Mink, 1992).

In addition of abilities of ilarviruses to infect deciduous trees, other ilarviruses infect non-woody plants; for example *asparagus (asparagus 2 ilarvirus,* Rafael-Martin and Rivera-Bustamante, 1999); strawberry=fragaria (*fragaria chiloensis (?) ilarvirus*; lilac, *lilac ring mottle ilarvirus,* Scott and Ge 1995); spinach (*spinach latent ilarvirus,* Scott *et al*., 2003); sunflower, *sunflower ringspot (?) ilarvirus,* tobacco (tobacco *streak ilarvirus*, Cornelissen *et al.*, 1984; Brunts *et al,* 1996). A newly discovered ilarvirus was reported on tomato by Marchoux *et al*. (1999).

Ilarvirus spp., *Bromovirus, Cucumovirus*, and *Alfamovirus*, belonging to the family *Bromoviridae,* have similar genome organization and encoding functionally translation products (Murphy *et al*., 1995).

Sugar beet (*Beta vulgaris* L) is an economic crop recently grown in Egypt. Its cultivation areas and productivity have been doubled several folds in the last 15 years. The total cultivated areas reached 153,711 feddans with an average of 20.6 tons/feddan (Anonymous, 2002).

In Egypt, sugar beet is infected with several viruses including *beet curly top* virus (Abdel-Salam and Amin, 1990), *beet yellows virus, beet western yellows virus,* and *cucumber mosaic virus* (Abdel-Salam *et al*., 1991), and *beet necrotic yellow vein virus* , the causing virus of rhizomania of sugar beet, (Abdel-Salam and El-Shazly, 2002). Recently, a disease with syndrome including ring spot, line pattern, brilliant mosaic, and with reduced growth has been observed on several beet plants in Kafr El-Sheikh, El-Giza, and El-Fayoum governorates. Such mentioned symptoms are mostly observed on stone fruits infected with ilarviruses (Fulton, 1983). The purpose of this study is to illustrate the nature of the causing virus to this phenomenon on sugar beet and discusses its relationship with *prunus necrotic ringspot virus* (*PNRSV*), a key member in the Genus: *Ilarvirus*.

MATERIALS AND METHODS

Virus isolation and propagation

The provisional name of *beet necrotic ringspot virus* (*BNRSV*) will henceforward be used to deal with the isolated virus from sugar beet for simplicity. Ringspot symptoms on beet seedlings grown in the Experimental Farm, Fac. Agric. Cairo Univ. were mechanically transferred onto sugar beet plants cv. Pleno grown in the greenhouse. The virus was purified biologically through three consecutive passages onto the local lesion host *Chenopodium quinoa* followed by one passage on the table beet (*Beta patellaris).* The resulting local ringspot were singly back inoculated onto *C. quinoa.* Finally, the systemic mottling symptoms formed onto *C. quinoa* plants were mechanically transferred on Pleno sugar beet plants for virus propagation.

Other used virus isolates and antisera

Isolates for *prunus necrotic ringspot virus* (*PNRSV*) were isolated and serologically induced by Dr. Aly M. Abdel-Salam (the first author in the present study) from the Experimental Farm, Fac. Agric., and Cairo Univ. The first isolate was isolated from peach and designated as *PNRSV*-PF, while the second was isolated from apricot, *PNRSV*-AP.

Transmission studies

1-*Mechanical transmission*

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 was used for mechanical transmission studies. Tested plants dusted with 600-mesh carborundum were inoculated with infective supernatant (diluted 1:3, v/v) with buffer. Inoculated plants were rinsed thoroughly and swiftly due to the corrosive nature of the STEP buffer.

2-Insect transmission

a) aphid transmission

Adults of the non-viruliferous green peach aphid (*Myzus persicae*) were fed on *BNRSV*-infected beet plants for 24 hr-acquisition access period (AAP). Insects were then transferred onto 30 healthy beet plants for 24-inoculation access period (IAP); using 10 insects per plant. Insects were then killed and plants were kept in an insect-proof greenhouse for symptom development.

b) Thrips transmission

Non-viruliferous onion thrips (*Thrips tabaci*) raised on onion plants were used in *BNRSV* transmission tests. Adult insects and first instar larvae were each fed (24 hr-AAP) onto *BNRSV*-infected beet leaves placed in yogurt plastic containers (8.5 cm, diameter and 4 cm, height). A rectangular shape (1.5x2.0 cm) was cut from the container lid. The cut area was replaced with *organza* cloth glued with silicon to prevent insects from escaping. The container was kept in a humid chamber during the time of the experiment. Insects were removed and transferred for 48 hr-IAP onto healthy Pleno cv. of sugar beet plants using 20 insects per plants. Plants in pots were kept under glass lantern covered from above with *organza* cloth glued with silicon. The insects were insecticide-sprayed afterwards and plants were maintained in the greenhouse for symptom development.

3-Seed transmission

Five commercial sugar beet cvs., namely Athospoly, Farida, Kawmera, Oscarpoly, and Pamela, were tested for virus transmission. Seeds of each cv. were seeded in plastic trays. Upon germination, 1000 seedlings/cv. were tested serologically with indirect ELISA using the induced antiserum (AS) for *BNRSV*.

Host range Studies

Different plant species were mechanically inoculated with *BNRSV*-infected sap and maintained for 45 days in the greenhouse for symptom development. Non-inoculated plants were left as a control. Some of the inoculated plants were serologically tested using *BNRSV*-AS.

Physical and chemical studies on purified virus

1-Virus purification

BNRSV was purified from infected Pleno cv. of sugar beet or gomphrena (*G. globosa)* according to the summation of several purification techniques for *PNRSV* (Fulton, 1959; Crosslin and Mink, 1992) with some modifications as illustrated in the flow chart in Fig.1.

2-Electron microscopy

Purified un-fractionated virus preparations of *BNRSV* were negatively stained with 2% phosphotungestic acid, pH 7.0, as described by Noordam (1973).

3-SDS-polyacrylamide gel electrophoresis

Analysis of molecular weight of virus coat proteins for *BNRSV* and *PNRSV*-PF were done according to Crosslin and Mink (1992) and Abdel-Salam *et al.* (2004).

4-Agarose gel electrophoresis

Extraction of RNA from purified *BNRSV* and *PNRSV*-PF was performed according to Crosslin and Mink (1992).

Serologic studies

1- Antiserum production

The pooled three components of the purified *BNRSV* from either sugar beet or *G. globosa* plants were used for the induction of

two-virus antisera. Antisera were induced in rabbit as described by Abdel-Salam *et al.* 2004) using 1 mg/ml purified virus/injection. The antiserum for *BNRSV* induced from sugar beet and *G. globosa* sources were designated as *BNRSV*-AS (B) and *BNRSV*-AS (G), respectively. The IgG fraction of *BNRSV*-AS was separated using the technique described by Mckinney and Parkinson (1987).

2- *Serologic tests*

a) Agar double diffusion test (ADDT)

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

b)Dot blotting immunobinding assay (DBIA) and tissue blotting immunobinding assay (TBIA)

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

c) *Direct ELISA*

Direct DAS-ELISA test was conducted as described by Clark and Adams (1977) to test serologic relationship between *BNRSV* and *PNRSV*.

Genomic studies

1-Primer selection

Two universal primers namely JC10 (antisense primer) and JC12 (plus-sense primer), derived from the sequence encoding the coat protein gene of *PNRSV* (Hammond *et al.*, 1998;1999) were used for reverse transcription polymerase chain reaction (RT-PCR). The oligonucleotide primers were synthesized by Metabion GmbH (Lena-Christ-Strasse 44, D-82125 Martinsried/ Deutscland).

2-Extraction of total RNA

Total RNA was extracted from *BNRSV*-infected sugar beet plants by applying the technique of *High Pure RNA Tissue Kit* (Roche Molecular Biochemicals, (Cat. No.2033674).

3-RT-PCR

RT-PCR running conditions were made as described by Hammond *et al.* (1998; 1999). The PCR cycles were repeated 35 times under the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The PCR products were analyzed by electrophoresis onto 1.0% agarose gels and 5% PAGE and the size of the full-length DNA fragment was determined in accordance with molecular weight markers. A fragment of 641 bp was amplified and cloned directly into pGEM-T-Easy cloning vector.

4-Restriction digestion of amplified RT-PCR product

The amplified RT-PCR product of *BNRSV* was digested by Mse I restriction endonuclease (Life Technologies Inc.) according to Hammond *et al.* (1998; 1999). The RT-PCR products were analyzed by electrophoresis on 5% polyacrylamide (PAGE) gel in 1X TBE buffer. After electrophoresis, the gel was stained with ethidium bromide and photographed. The restriction digestions were analyzed by electrophoresis on 3 % Nuseive agarose gel.

5-Immunocapture- reverse transcription-polymerase chain reaction (IC-RT-PCR)

IC RT-PCR using single-tube and buffer for the reaction was performed as described by Benoit Moury *et al.* (2000).

6- Molecular cloning

PCR product was purified from the low melting agarose gel using GFX Agarose Gel Extraction Kit (Amersham, Pharmacia, and Uppsala.) and ligated into a pGEM-T Easy plasmid vector system supported from 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 recombinant *BNRSV* plasmids. The recombinant DNA plasmids containing the coat protein (CP) gene of *BNRSV* was purified from transformed cells using the High Pure plasmid preparation kit (Roche). Successful clones were verified by PCR using successive M13F/M13/R and JC10/JC12 primer pairs and southern hybridization.

7- Automated DNA sequencing:

DNA sequencing of the amplification products was performed using CLIP, a sensitive sequencing method developed by Visible Genetics Inc. Each sequencing reaction was loaded on a long read tower (Visible Genetics Inc.), an automated DNA sequencer. The resulting sequence for each sample was then compared to a database containing known *PNRSV* isolates using the Clustral W method (Felsenstein, 1985). A phylogenetic analysis of a 641 nucleotide segment of the *PNRSV*/CP was performed on all of the sequences generated in this study (Olsen *et al*., 1994). Ilarviruses sequences obtained from GenBank were included in this analysis. The resulting PCR product of 641 bp was subsequently purified by Qiagen spin column and precipitated by ethanol. The purified product of 641 bp was subsequently purified and sequenced by the dideoxynucleotide method with the automated clipper sequencer (Visible Genetics). Amplification of negative samples with PCR in the coat protein region produced no sequence. To avoid contamination, all analyses were performed following Kwok and Higuchi's recommendations (Kwok and Higuchi, 1989).

RESULTS

Biological and biochemical studies

Field symptoms

Starting symptoms include small pale to yellow ringspots on small sugar beet plants. Upon disease progress, plants develop variable symptoms including callico mosaic with large brilliant yellow areas on the leaf margins (Fig. 2).Line pattern can also be seen. The described symptoms are seen sporadically in the infected plants. Upon aging, infected plants show recovery from symptoms.

Transmission studies

1-Mechanical transmission

BNRSV was transmitted easily to sugar beet seedlings in the greenhouse. Symptoms appeared seven to ten days post inoculation Inoculated plants showed chlorotic and necrotic ringspots followed by yellow mosaic and line pattern on the successive leaves (Fig. 2).

2-Insect transmission

a)aphid transmission

Trials made for transmitting *BNRSV* by *Myzus persicae* failed. All plants had negative indication in both biological (symptoms) and serological (ELISA) tests.

b) Thrips transmission

Thrips tabaci insects were able to transmit *BNRSV* from infected to healthy beet seedlings. Infected seedlings showed ringspot symptoms after one month from inoculation. The results were verified with RT-PCR and IC-RT-PCR analysis. Results showed that *BNRSV* was present in both adult insects and larvae (first instars), but not in second instars and pre-pupa larvae (Fig. 15).

3) *Seed transmission*

ELISA testing of 5000 seedlings from five commercial different sugar beet cvs. (namely, Athospoly, Farida, Kawmera, Oscarpoly, and Pamela) indicated that *BNRSV* was not seed-transmitted in these varieties.

Host range studies

Results in Table (1) and Fig. (3) indicated that *BNRSV* had a wide host range. There were at least 21 hosts belonging to

seven families were susceptible when tested using differential hosts, TBIA, DBIA, and ELISA tests. The induced symptoms on the tested hosts ranged between ringspots, color breaking, oak leaf pattern, line pattern, and shoot holes (Fig. 3). Interestingly, *BNRSV* was able to induce similar symptoms to those induced by the authentic isolates of *PNRSV* when infecting goose foot, rose, apricot and peach plants.

Physical and chemical studies on purified virus

1-Virus purification

BNRSV migrated in the sucrose density gradients (SDG) forming three components, i.e. top (T), middle (M), and bottom (B), (Fig. 4). The average $A_{260/280}$ ratio for these components, either purified from sugar beet or gomphrena plants, was 1.63. Unfractionated preparations either measured before or after SDG (pooled preparations of T, M, and B) of *BNRSV* and *PNRSV*-PF, both purified from gomphrena plants, had A $_{260/280}$ ratios of 1.5-1.55 and 1.55-1.6, respectively (Fig. 5). Either *BNRSV* or *PNRSV*-PF had A max and A min at 260 nm and 240 nm, respectively. Yield of purified *BNRSV* from gomphrena plants was 0.75 mg/g tissue.

2-Electron microscopy

Electron microscopy of the purified virus preparation, negatively stained with 2 % phosphotengestic acid revealed isometric virus particles with 23 nm in diameter as shown in Fig. (6).

3- SDS-polyacrylamide gel electrophoresis (PAGE)

Molecular weight determination of coat protein subunits for both *BNRSV* and *PNRSV*-PF was *ca.*29 kDa for both viruses when determined with SDS-PAGE (Fig. 7).

4-Agarose gel electrophoresis

Both purified *BNRSV* and *PNRSV*-PF were separated into four RNA components in agarose-gel electrophoresis upon treatment with 10% SDS (Fig. 8).

Serological studies

Serologic relationship between BNRSV and PNRSV

ADDT revealed serological homogeneity between purified *BNRSV* and *PNRSV* isolates (peach and Apricot) and their respective antisera (Fig. 9). Similarly DBIA results showed cross reactivities between *PNRSV*-AS (Sanofi) and *BNRSV*-AS upon reaction with purified virus preparations of *BNRSV* and *PNRSV* isolates from peach and apricot (Fig. 10). *BNRSV*-AS was also able to detect *PNRSV* from infected peach trees upon using DAS-ELISA (Fig. 11).

Detection of BNRSV in infected plants and viruliferous thrips insects

IC- RT-PCR successfully detected *BNRSV* in viruliferous thrips insects. Only adults and 1st instar larvae carried the virus (Fig.15). IC-RT-PCR detected also *BNRSV* in sugar beet leaves exposed to viruliferous thrips.

Genomic studies

Detection of PNRSV using RT-PCR

Results in Fig. (12) showed that universal primers for ilarviruses JC10 and JC12 were able to amplify products of 641 bp of coat protein of *BNRSV* from sugarbreet as well as from *PNRSV*-PF.

Restriction digestion of amplified RT-PCR product

The amplified PCR products (641 bp) of *BNRSV* was digested by an Mse I restriction endonuclease and migrated on agarose gel electrophoresis forming two bands with 313 bp and 328 bp (Fig. 13).

Sensitivity of RT PCR and IC RT-PCR for the detection of BNRSV in T.tabaci insects

Results in Fig. (14) showed the ability of RT-PCR and IC-RT- PCR to detect *BNRSV* in both the adults and 1st instar larvae of thrips insects. IC-RT-PCR; however, was more sensitive than RT-PCR. On the other hand, neither test was able to detect the virus in 2nd instar larvae.

Multiple alignment of the nucleotide sequences and Phylogenetic analysis

Comparison of the nucleotide sequence and multiple alignments of *BNRSV-EG* and *PNRSV* isolates (Fig. 16&17) showed extensive conservation (~ 90%) identity) among isolates of *PNRSV* (93.15 %) to the AF465235 (*prunus necrotic ringspot virus* isolate CH3 coat protein gene); 91.03 % to the AF465236 (*prunus necrotic ringspot virus* isolate CH19 coat protein gene), 89.14 % to AF465232 (*prunus necrotic ringspot virus* isolate ring 28 coat protein gene), 90.47 % to the AF465225 (*prunus necrotic ringspot virus* isolate ring 21 coat protein gene). With other ilarviruses, *BNRSV*-EG coat protein sequence shared approximately 67.30 % identity with the CPs of *asparagus virus II, tobacco streak virus, and spinach latent virus ilarvirus* (Fig. 17).

DISCUSSION

The present study is describing the presence of a newly unreported illarvirus, i.e. *Beet necrotic ringspot virus, BNRSV,* on sugar beet in Egypt. This drown statement was built on results of comparatively extensive experiments dealing with biological, biochemical, physical, and molecular aspects on the virus under study as well as on isolates of *PNRSV* infecting peach (*PNRSV*-PF) and apricot (*PNRSV*-AP).

The name of *BNRSV* has been suggested due to its induced symptoms on sugar beet as well as on other tested hosts (Figs. 2 &3). These symptoms mimic similar ones induced by the rugose mosaic isolates of *PNRSV*. *BNRSV* was found to infect a range of hosts (Table 1 and Fig. 3) that resemble those reported by some *PNRSV* isolates especially in the genera of *Chenopodium, Gossypium, Gomphrena, Nicotiana, and Prunus* (Fulton, 1957; Kirkpatrick *et al.*, 1967). Inoculated peach and apricot plants with *BNRSV* had symptoms typical to those produced by *PNRSV* on these two hosts (Fig. 3-F, G). However, some differences in host reactions were also observed. For instance, no chlorotic lesions were observed on inoculated cucumber (*Cucumis sativus*) with *BNRSV*; instead line pattern was observed (Table 1). Additionally, *PNRSV* is not reported infecting sugar beet experimentally (Brunts *et al.*, 1996). Mechanical inoculation of *PNRSV*-PF on sugar beet resulted in chlorosis and leaf malformation (Fig. 3-K), which differed from those symptoms produced by *BNRSV* on sugar beet. Nevertheless, it should be kept in mind that *PNRSV* has different pathotypes varying in their induced symptoms (Aparicio and Pallas, 2002). Sugar beet, on the other hand, is susceptible to infection with three ilarviruses, viz. *asparagus 2 ilarvirus, spinach latent ilarvirus,* and *tobacco streak ilarivurs* (Brunts *et al.*, 1996). However, results of tested hosts in Table (1) when compared with the described hosts for these viruses (Brunts *et al.*, 1996) indicate some differences in host reactions.

In addition to the proven mechanical transmission nature of *PNRSV* and *BNRSV* (Table 1), *PNRSV* has been known as a seed-transmitted virus (Brunts *et al.*, 1996). However, *BNRSV* was proven not to be seed-borne at least in the five tested sugar beet cvs.

The roles played by thrips vectors, including *Frankineilla occidentalis* and/or *Thrips tabaci*, in transmitting the pollen-borne

ilarviruses *PNRSV* and *PDV* (Greber *et al.* 1991, 1992) and *tobacco streak virus* (Sdoodee and Teakle, 1993) have been confirmed (Ullman *et al.*, 1997). The obtained results showed that the insects of *T. tabaci* were able to acquire and transmit *BNRSV* from infected to healthy sugar beet seedlings respectively under greenhouse conditions. RT-PCR and IC-RT-PCR (Fig.15) were able to detect the *BNRSV* inside the 1st instar and adult insects, but not 2nd instar larvae. Such failure of virus detection is due to the fact that only 1st instar larvae can acquire the virus, while pupal stages of thrips are non-feeding (Ullman *et al.*, 1997). IC-RT-PCR was able to detect *BNRSV* in symptomatic sugar beet leaves previously exposed to viruliferous *T. tabaci;* an indication of positive role played by thrips in transmitting *BNRSV* (Fig.15).

Purified *BNRSV* separated in SDG into three components (top, middle and bottom), (Fig. 4) resembling ilarviruses as reported by Fulton (1967) and Gonsalves and Fulton, (1977) and Crosslin and Mink (1992). Serologic testing of these components in SDG (Fig. 10) indicated that most of serologic activities were located in the middle and bottom components; indicating that the top component fraction may contain virions with poor immunogenic or defected coat protein. Interestingly, Fulton (1967) found that the top component of RMV was not infectious. By comparing our results with those of Fulton (1967) and considering the genomic activation phenomenon by the coat protein of ilarviruses (Aparacio and Pallas, 2002), it appears that integrity of the coat protein is of prime importance for infectivity.

Electron microscopy of purified *BNRSV* particle showed isometric particles with 23 nn in diameter (Fig. 6) which agrees with measures reported by Brunts *et al.* (1996) for *PNRSV*.

The average $A_{260/280}$ ratios of the three fractionated and un fractionated components were 1.63 and 1.5, respectively. These results comply with similar ratios of 1.43-1.67 for fractionated and 1.5 for un-fractionated purified ilarviruses reported by Gibbs (1988).

The measured molecular weight of the coat protein subunits of both *BNRSV* and *PNRSV*-PF was 29 kDa (Fig. 7); which is within the values reported for *PNRSV* by Crosslin and Mink (1992).

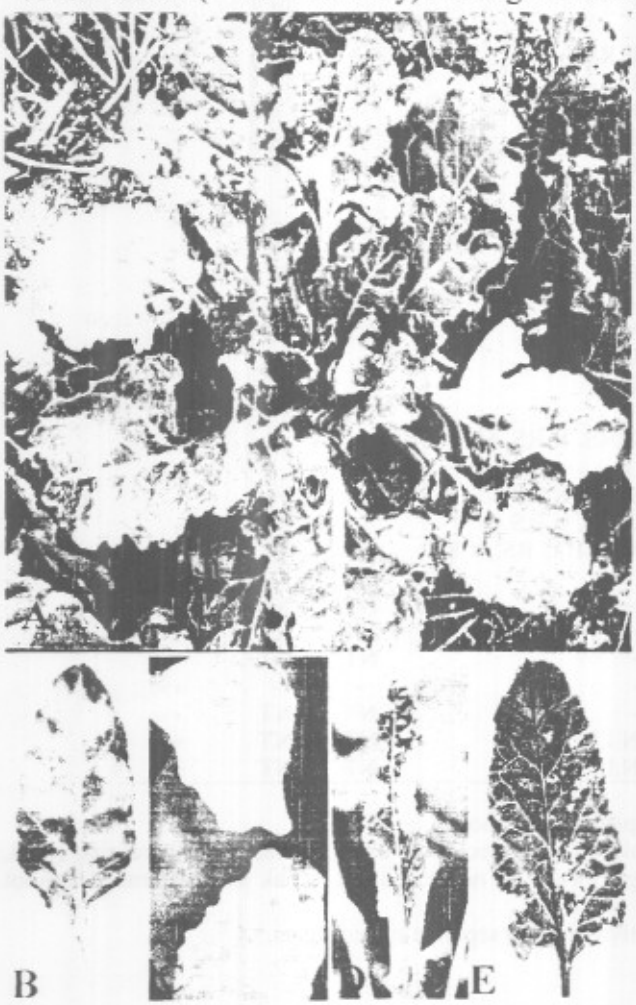
Genomic RNAs of *BNRSV* and *PNRSV*-PF migrated in agarose gel forming four species of RNA (Fig. 8). Such results were typical to the properties of *PNRSV*-RNAs and other ilarviruses as stated by Gonsalves and Fulton (1977) and Sánchez-Navarro and Pallas (1997).

Serologic studies indicated cross reactivity between the tested antisera of *PNRSV* (the one from France and the locally induced one to *PNRSV*-PF) and that of *BNRSV* when examining these antisera against antigens of *BNRSV*, *PNRSV*-PF, and *PNRSV*-AP using DDBIA and ADDT (Figs. 9 &10). Further *BNRSV*-AS was able to detect *PNRSV* in infected peach trees with DAS-ELISA (Fig. 12). These collective results indicated close serologic ties between *BNRSV* and *PNRSV*, which suggest putting them in the same serotype as described by several authors working on typing serotypes of ilarviruses (Barbra *et al.*, 1978; Crosslin and Mink; 1992, Mink, 1987; Spiegel *et al.*, 1999; Vaskova *et al.*, 2000; Scott *et al.*, 2003).

The universal primers JC10/JC12 for *PNRSV* (Hammond *et al.* 1998) were able to amplify 641 bp of the coat protein genes of both *BNRSV* and *PNRSV*-PF (Fig. 13) indicating the nature of *BNRSV* as an ilarvirus. Further, positive restriction digestion of the amplified 641 bp of *BNRSV* with Mse I (Fig. 14) indicated the presence of an Mse I restriction site in the coat protein gene of

indicating the nature of *BNRSV* as an ilarvirus. Further, positive restriction digestion of the amplified 641 bp of *BNRSV* with Mse I (Fig. 14) indicated the presence of an Mse I restriction site in the coat protein gene of *BNRSV*-RNA3: resembling, therefore, the rugose (severe) isolates of *PNRSV* (postive to Mse I). Mild isolates of *PNRSV*, on the other hand, are negative to Mse I digestion (Hammond *et al*. 1998; Hammond *et al. 1999)*.

Comparison of the partial nucleotide sequence of *BNRS*V-EG show extensive conservation (~ 90% identity) among isolates of *PNRSV* reported by Hammond, (2003). *BNRSV*-EG coat protein sequence shared approximately 67.30 % identity with the CPs of other ilarviruses, viz. *asparagus virus II, tobacco streak virus* and *spinach latent virus ilarvirus* (Fig. 16 &17). These obtained results in addition to results of ADDT (Fig. 9) and DBIA (Fig. 10), suggest that *BNRSV* can be grouped under the same serotype. Differences in some host range, especially sugar beet, indicated that *BNRSV* and *PNRSV* are probably different pathotypes for the same virus.



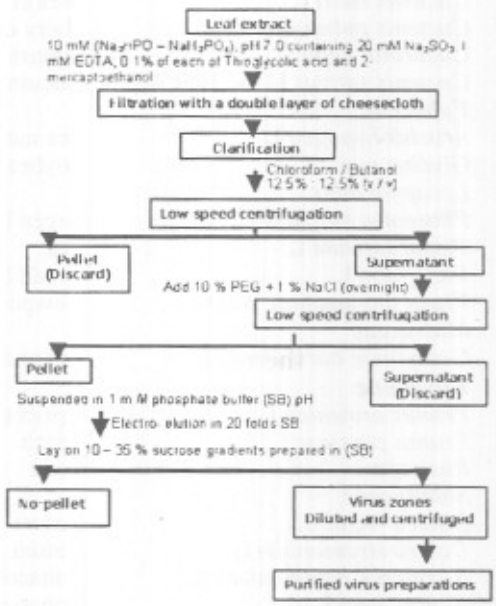


Fig. (1): Flowchart showing the purification steps followed for purifying BNRSV and PNRSV.

Fig. (2): Symptoms of an infected sugar beet plant with BNRSV in a commercial sugar beet field in Kaf El-Shiek governorate. B-E, a follow up of symptom development in the greenhouse upon the mechanical inoculation of sugar beet plants cv. Pleno with BNRSV. B, D, chlorotic and necritic ringspot, respectively; D, E, development of brilliant mosaic on following leaves.

Table (1): Host range of beet necrotic ring spot ilarvirus tested by mechanical inoculation.

Test plant*	Common name	Observed Symptoms	TBIA	DBIA	ELISA
Amarnthaceae					
Gomphrena globosa L.	Gomphrena	C.RS.	+	+	+
Chenopodiaceae					
Beta vulgaris L.	Sugar beet	C.RS, N.RS, LP, M	+	+	+
Beta vulgaris var.*Cicla* L.	Swiss chard	C.RS, N.RS, OLP	+	+	+
Beta patelaris L.	Table beet	L.RS	+	+	+
C. amaranticolor Coste & Reyn	Gooes foot	N.LL	NT	NT	NT
Chenopodium murale	Gooes foot	N.LL	NT	NT	NT
C. quinoa Willd	Gooes foot	N.LL, Sys.Mot.	NT	NT	NT
Compositae					
Hilianthus annuus L.	Sun flower	-	-	-	-
Lactuca sativa L.	Lettus	C.RS.	-	+	+
Xanthium brasilicum V.	Broad cocklebur	M. C.RS.	+	+	+
Zinnia elegans Jacq	Golden zinnia	-	-	-	-
Cuccurbitaceae					
Citrullus vulgaris L.	Water melon	E.RS	-	-	-
Cucumis melo L.	Melon	LP.	+	+	+
Cucumis pubescens L.	Hairy cucumber	LP.	-	+	+
Cucurbita pepo L.	Squash	LP.	+	-	-
Cucumis sativus L. cv. Beit Alpha	Cucumber	LP.	+	+	+
Fabaceae					
Arachis hypogea L.	Pea nut	-	-	-	-
Glycine max L.	Soybean	-	-	-	-
Lucaenia alba		LP		+	
Phaseolus vulgaris L.	Green bean	-	-	-	-
Pisum sativum L.	Pea	Vy.	NT	NT	NT
Vicia faba L.	Broad bean	LP.	-	-	+
Vigna unguiculata Walp..	Cowpea	LP.	+	-	-
Malvaceae					
Gossybium barbadense L	Egyptian cotton	C.RS, N.RS, SH	+	+	NT
Rosaceae					
Prunus armenica L.	Apricot	C.RS, N.RS	-	+	+
Prunus persicae L.	Peach	C.RS, N.RS	-	+	-
Rosa spp.	Rosa	Vy,CB,C.RS, N.RS	+	-	+
Solanaceae					
Datura metal L.	Datura	-	NT	NT	-
Datura stramonium L.	Datura	-	NT	NT	-
Nicotiana. benthamiana L.	Tobacco	-	NT	NT	-
N. glutinosa L.	Tobacco	-	-	-	-
N. rustica L.	Tobacco	-	NT	NT	-
N. tabacum L. cv.Java	Tobacco	N.LL	NT	NT	+
N. tabacum L. cv. White Burley	Tobacco	N.LL	NT	NT	+

*= tested plants were back inoculated on *C. murale* using four plants/tested host.
CB=flower color breaking, E.RS= etched ring spots, C.RS=chlorotic ring spots, LP=line pattern, L.RS=local ring spots, M=mosaic, , N.LL=necrotic local lesions, N.RS=necrotic ring spots, NT= not tested, OLP=oak leaf pattern, SH=shoot holes; Sys. Mot=systemic mottling, VY=Vein yellowing.
Ten plants from each tested plant species were tested for infectivity in two separate experiments.

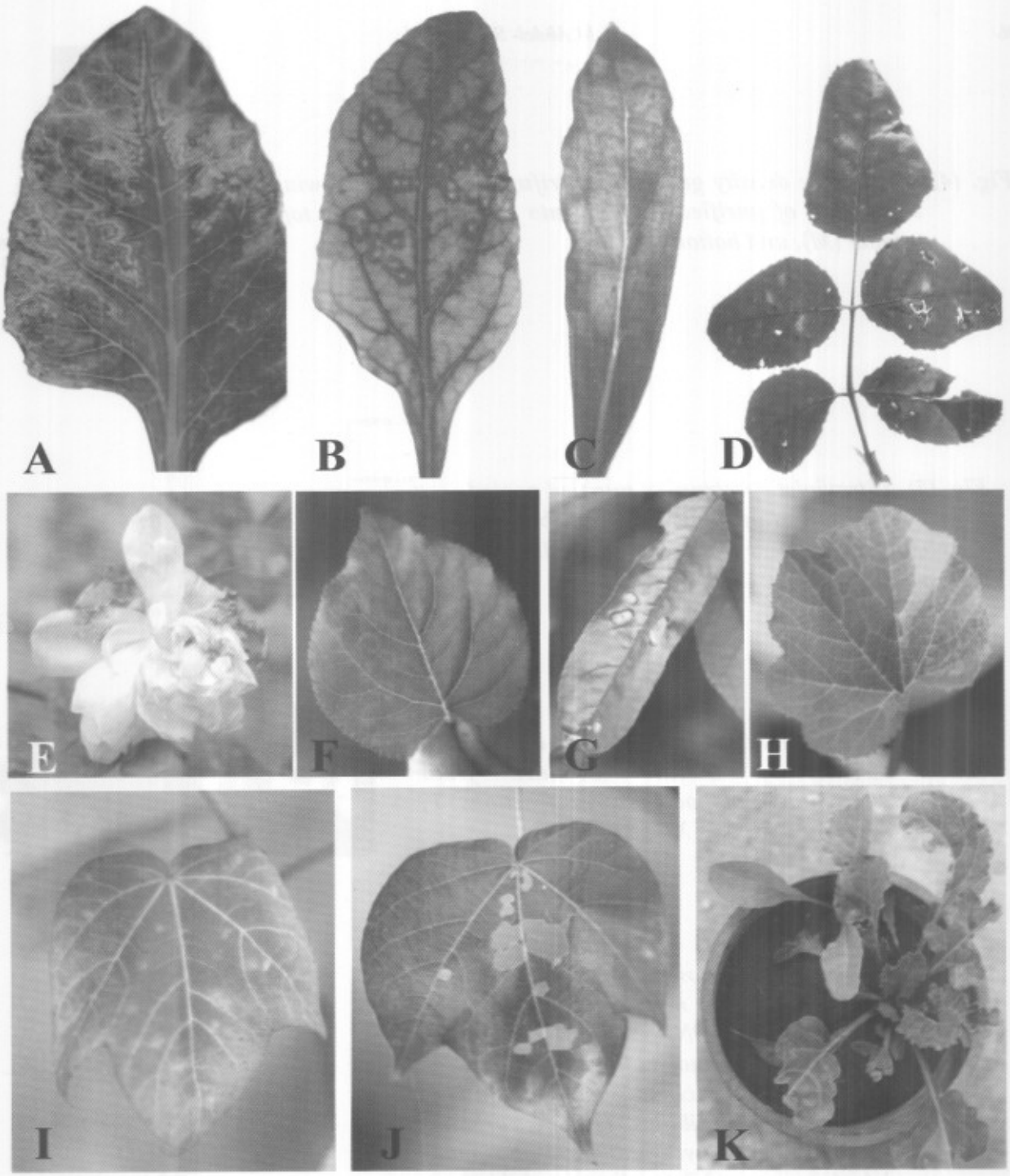


Fig. (3).Symptoms of BNRSV infection on some hosts upon mechanical inoculation. A, swiss chard (B. vulgaris Cicla) showing oak leaf patern; B, C, and D, leaves of table beet (B. patellaris), G. globosa, Rose sp. showing ringspots; E, a rose flower showing malformation and color breaking; F,G apricot and peach leaves showing chlorotic and necrotic ringspots, respectively; H, a squash leaf with line pattern; I,J cotton leaves showing chlorotic ringspots and shot holes symptoms;K, a sugar beet plant cv.Pleno inoculated with PNRSV-PF from an infected peach tree showing chlorosis and leaf malformation.

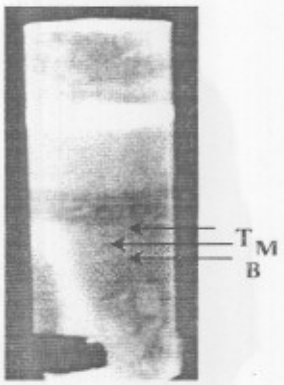


Fig. (4): A Sucrose density gradient centrifugation output showing the separation of purified BNRSV into three components top (T), middle (M), and bottom (B).

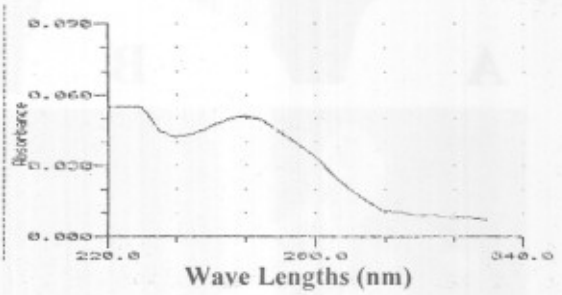


Fig. (5). Ultraviolet spectrum of purified pooled fractions (top, middle, and bottom) after recovery from sucrose gradients.

Fig. (6). Electron microscopy testing of Purified virions of BNRSV obtained from examining the pooled three fractions T, M,and B. Mag. = X 150,000

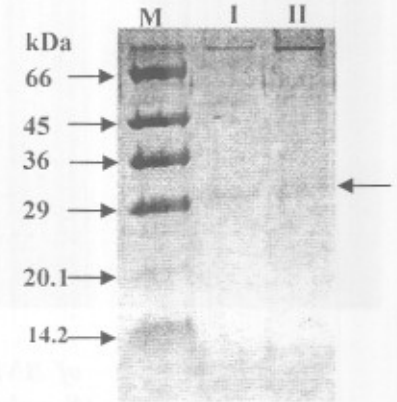


Fig. (7): SDS-PAGE showing similarities in coat protein subunit molecular weights of BNRSV (I) and PNRSV-PF (II) indicated by the arrow. M, molecular weight Markers (Sigma) in kDa including lactalbumin (14, 2), Trypsin inhibitor (20.1), carbonic anhydrase (29), glyceraldhyde-3-phosphate dehydrogenase (36) Ovalbumin chiken egg (45), Albumin bovine serum (66).

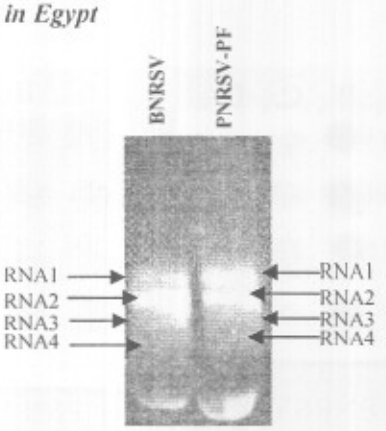


Fig. (8): Agarose gel electrophoresis showing the migration of genomic RNAs of BNRSV and PNRSV into four RNA species (RNA 1 -4) after treatment of their respective virions with SDS.

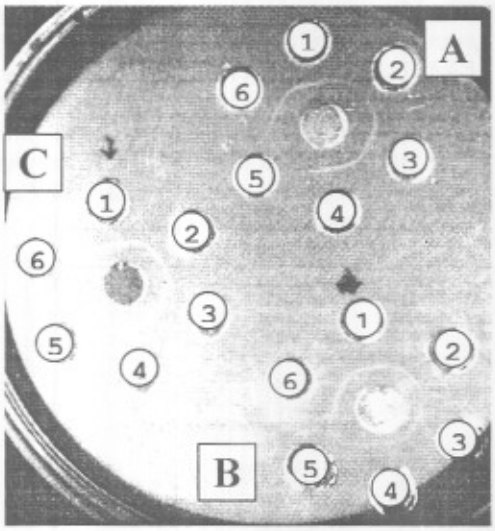


Fig. (9): Agar double diffusion test showing the serologic relationships between BNRSV and PNRSV isolates from peach (PNRSV-PF) and apricot (PNRSV-AP). Central wells contain 1/10 diluted antisera induced for A, PNRSV-PF purified from G. globosa ; B, BNRSV purified from sugar beet; C, BNRSV purified from G. globosa. Peripheral well contains 50 ug, each, of purified BNRSV (1, 3), PNRSV-PF (2), PNRSV-AP (3, 6), 4, healthy control (G. globosa).

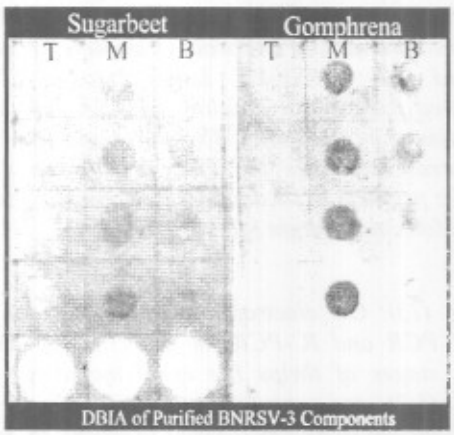


Fig. (10): DBIA showing the serologic reactions of BNRSV-AS with each of the three facrtions T, M, and B of purified BNRSV from sugar beet and gomphrena plants. BNRSV-AS was used at 1/1000 dilution and goat antirabbit alkaline phosphatase conjugate at 1/5000 dilution. NBT/BCIP complex was used for color development. The last rows in both membranes represent healthy sugar beet and gomphrena plants.

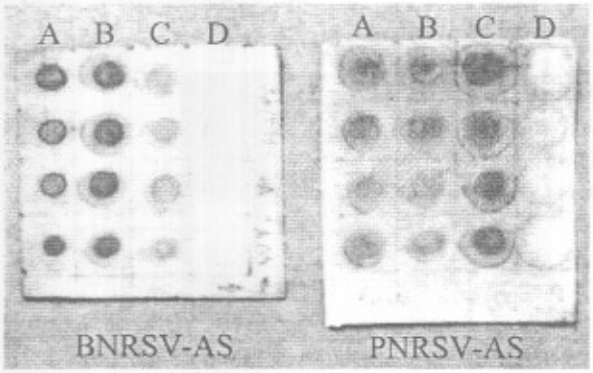


Fig. (11). DBIA showing the cross serologic reactivities between BNRSV-AS and PNRSV-AS (Sanofi) with purified (1 µg/square) tested preparations of BNRSV (A), PNRSV-PF(B), and PNRSV-AP(C) isolates. Both antisera were used at 1/1000 dilution. Secondary antiserum of goat antirabbit alkaline-phosphatase conjugate was used at 1/5000 dilution. D, represent healthy control of G. globosa. Naphthol As-MX/Fast Red TR were used as cromogenic substrates.

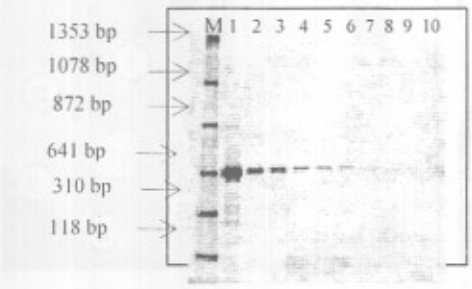


Fig. (13): 5% polyacrylamide gel electrophoresis (PAGE) showing the RT-PCR products amplified from the total-RNA extracted from sugar beet tissues infected with BNRSV. Lane 1: Positive control RT-PCR product amplified from prunus flower infected with PNRSV-PF. Lanes 2 to 9: RT-PCR products amplified from serial dilutions of RNA extracted from sugar beet tissues infected with BNRSV. Lane 10: Healthy control. The arrow indicates the PCR product amplified using the specific primers JC10 and JC12 (Expected size: 641 bp). M: DNA Molecular weight Marker (Roche, IX).

Fig. (15): Gel electrophoresis showing the sensitivity of RT-PCR and IC-PCR for detection of BNRSV in different stages of thrips life cycle including: 2nd instar larvae (1,3), pre-pupa larvae (2,4), adults (5,7), and 1st instar larvae (6,8). H, Non-viruliferous insects collected from onion plants. M: molecular weight standard (1 kb DNA ladder, Sigma)

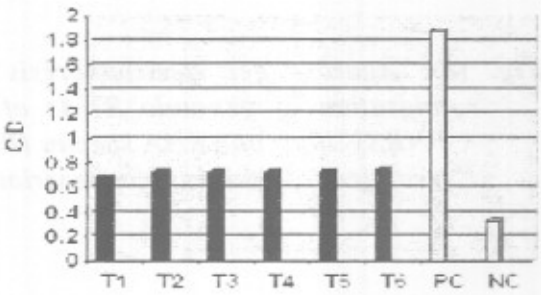


Fig. (12) DAS-ELISA showing the ability of BNRSV-AS to detect PNRSV in infected peach trees (T, six trees) in the experimental farm of Cairo University. PC=positive control of BNRSV-infected sugar beet; NC= healthy sugar beet.

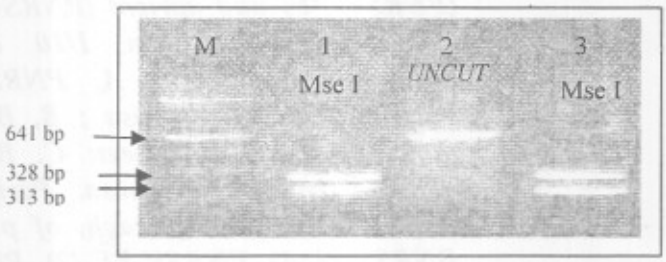
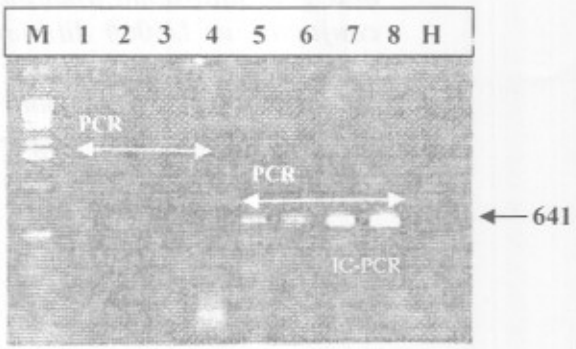


Fig. (14). The electrophoretic pattern of restriction digests of the JC10/JC12 PCR products amplified from BNRSV-infected sugar beet leaves. Lane M: Molecular weight marker (Roche), Lanes 1 and 3: Mse I restriction digestion products of the JC10/JC12 RT-PCR product of expected products size (313bp and 328bp). Lane 2: Uncut RT-PCR product amplified by using the JC10/JC12 primer pair of expected size (641bp).



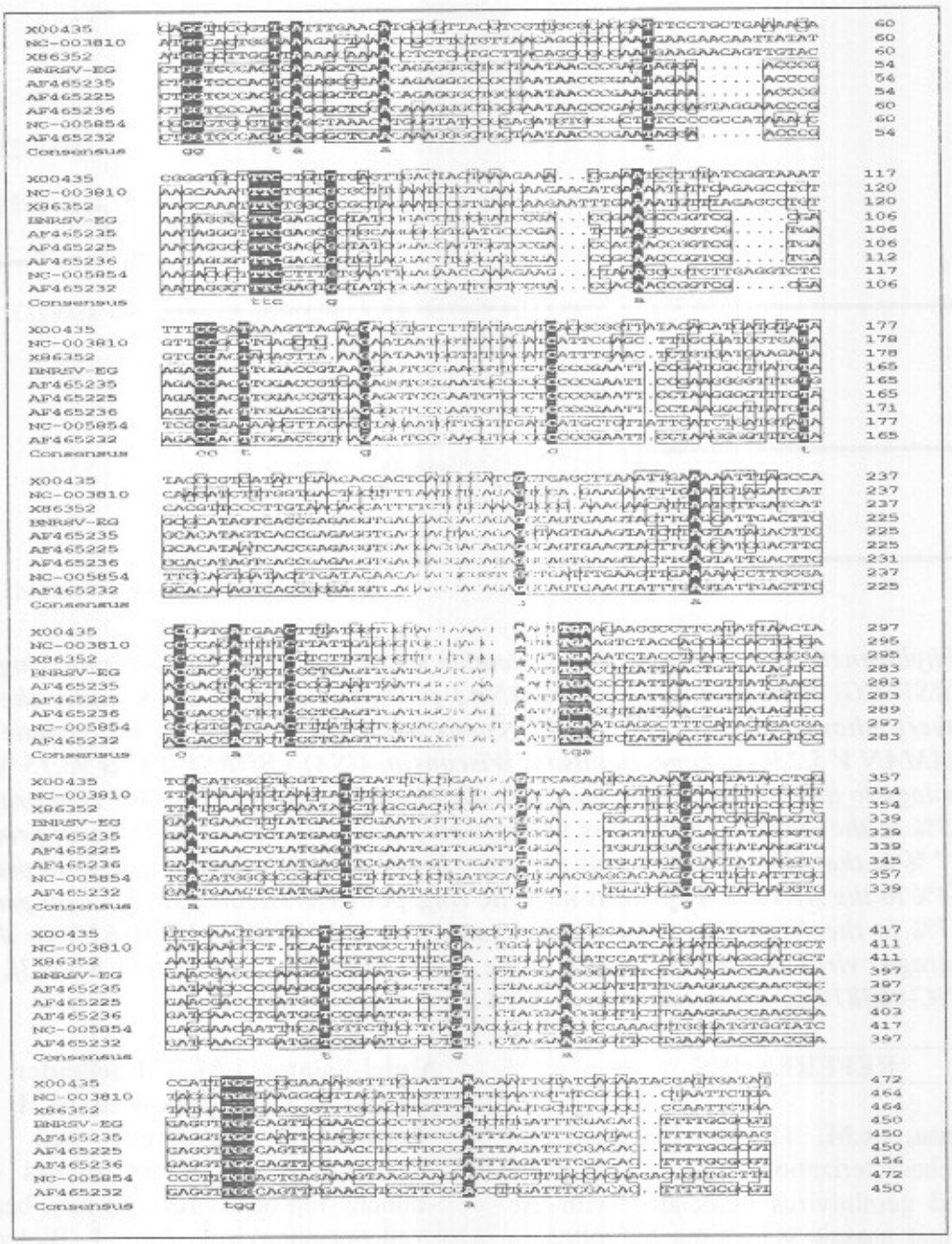


Fig. (16): A multiple alignment of partial nucleotide sequences encoding the coat proteins (RNA3) of the published ilarviruses with the Egyptian isolate of BNRSV-EG. X00435: tobacco streak virus RNA 3; NC-003810: spinach latent virus RNA 3; X86352: asparagus virus II RNA3; AF465235: prunus necrotic ringspot virus isolate CH3 coat protein gene; AF465225: prunus necrotic ringspot virus isolate Ring21 coat protein gene; AF465236: prunus necrotic ringspot virus isolate CH19 coat protein gene; AF465232: prunus necrotic ringspot virus isolate Ring 28 coat protein gene; and NC-005854: parietaria mottle virus. BNRSV-EG isolate showed 65.80% identity with the published ilarviruses. The alignment was generated using (DNAMAN V 5.2.9 package, Madison, Wisconsin, USA). Identical nucleotides are surrounded by black boxes. Mse I restriction sites (T↓T A A) are located between the base nucleotide sequence in BNRSV-EG (268-269), AF465235: prunus necrotic ringspot virus isolate CH3 (268-269), AF465236: prunus necrotic ringspot virus isolate CH19 (268-269).

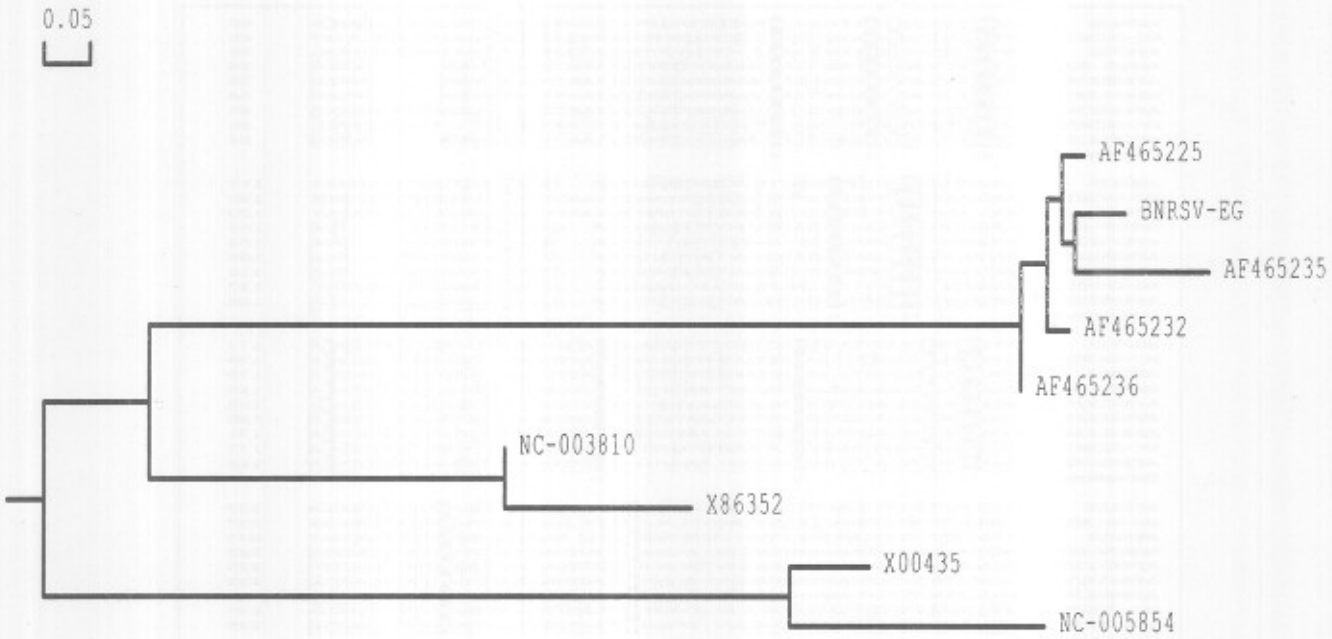


Fig.(17): Phylogenetic tree of the partial nucleotide sequences of the beet necrotic ring spot virus (BNRSV-EG) RNA3 and ilarviruses RNA3 published in gene bank under the accession numbers shown above. The phylogenetic analysis was based on genetic distances (DNAMAN V 5.2.9 package, Madison, Wisconsin, USA). BNRSV-EG is 93.15 % sequence homology to the AF465235 (prunus necrotic ringspot virus isolate CH3 coat protein gene), 91.03% to the AF465236 (prunus necrotic ringspot virus isolate CH19 coat protein gene), 90.47 % to the AF465225 (prunus necrotic ringspot virus isolate ring 21 coat protein gene, 89.14% to the AF465232: prunus necrotic ringspot virus isolate Ring 28 coat protein gene, 41.77% to the NC-005854 (parietaria mottle virus, PMV RNA3), 40.63% to the X86352 (asparagus virus II RNA3), 39.03% to the X00435 (tobacco streak virus RNA 3), 23.98% to the NC-003810 (spinach latent virus RNA 3).

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المجلة العربية للتكنولوجيا الحيوية

تتلخص أهداف هذه المجلة في

- تشجيع التعاون العلمي بين العلماء من شتى أقطار الوطن العربي ونظرائهم من مختلف الدول .
- الإعلام على المستوى العالمي عن مختلف مجالات البحوث الجارية في العالم العربي ، في هذا المجال الحيوي .

الاشتراك السنوي

- للهيئات ومراكز البحوث والجامعات : 100 دولار أمريكي أو ما يعادلها بالعملة المصرية شاملة مصاريف البريد .
- للأفراد : 25 دولار أمريكي أو ما يعادلها بالعملة المصرية شاملة مصاريف البريد .
- متوافرة علي أقراص إلكترونية (CD) : 10 دولار أمريكي أو ما يعادلها بالعملة المصرية شاملة مصاريف البريد .

ملحوظات

- الآراء التي ترد في المقالات المنشورة تعبر عن رأي أصحابها فقط ولا تعبر عن وجهة نظر المجلة العربية للتكنولوجيا الحيوية .
- لا يسمح بإعادة طبع أي جزء من المجلة أو نسخه بأي شكل وبأي وسيلة سواء أكانت إليكترونية أو آلية ، بما في ذلك التصوير والتسجيل أو الإدخال في أي نظام حفظ أو استعادة معلومات إليكتروني ، بدون الحصول على موافقة كتابية مسبقة من إدارة المجلة .

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الملخص العربي

دراسات بيولوجية، بيوكيميائية، سيرولوجية، وجينية على فيروس البقع الحلقية الميتة فى البنجر: احد الفيروسات الحديثة التابعة لمجموعة الـ Ilarvirus والتى تصيب بنجر السكر فى مصر

على محمد مأمون عبد السلام*، منال على الشاذلى ، و هيام سامى عبد القادر****

*قسم امراض النبات، كلية الزراعة، جامعة القاهرة، الجيزة، 12613، مصر.

**قسم بحوث الفيروس، مركز البحـ ـث الزراعية، الجيزة، 12619، مصر.

تم عزل احد الفيروسات التى تصيب بنجر السكر لاول مرة فى مصر. وقد اعطى الاسم " البقع الحلقية الميتة فـى بنجر السكر Beet necrotic ringspot virus,BNRSV . ويسبب هذا الفيـروس مظهـر الحلقـات والطـرز الخطـى والموزيك الاصفر على نباتات البنجر فى الحقل. وبينت دراسات العدوى قدرته على اصابة نباتات تتبع سبع عائلات نباتيـة. وبينت دراسات النقل التجريبى انتقال BNRSV ميكانيكيا وبواسطة حشرة تربس البـصل Thrips tabaci ، ولكـن لـيس بواسطة حشرة من الخوخ Myzus persicae. كذلك بينت الدراسات عدم انتقال BNRSV خلال بذور خمـسة اصـناف مختبرة من البنجر. ووجد ان الفيروس المنقى يهاجر فى اعمدة السكروز المتدرجة منفصلا الى ثلاثة مكونـات. ووجـد ان متوسط نسبة الامتصاص A260/280 للمكونات المفصولة والغير مفصولة تساوى 1.5 و 1.63 على التوالى. كذلك وجد ان وحدات الغلاف البروتينى للفيروس تساوى 29 كيلو دالتون. وامكن فصل مكونات الحمض النووى الى اربعـة مكونـات بواسطة الهجرة الكهربية فى الاجاروز. وبينت دراسة البيولوجيا الجزيئية التى تمت على الجينوم الفيروسى قـدرة البادئـات JC10/JC12 الخاصة بفيروس البقع الحلقية الميتة فى البرقوق prunus necrotic ringspot Virus, PNRSV والتـابع لمجموعة Ilarvirus على بلمرة 641 bp من جين الغلاف البروتينى لهذا الفيروس، وكذا لفيروس PNRSV . كذلك وجد ان جين الغلاف البروتينى لـــ BNRSV يحتوى على مواقع حساسة لـلـــ Mse I –restriction enzyme ومـشابها بذلك العزلات الشديدة لـ rugose mosaic isolates لفيروس PNRSV . وقد اوضحت دراسات التتـابع النيوكليوتيـدى لجزء من جين الغلاف البروتينى لـ BNRSV وجود نسبة تشابه تصل الى 65% مع أربعة فيروسـات مـن مجموعـة Ilarvirus المنشورة فى بنك الجينات و أيضا تم مناقشة درجة التشابه و القرابة بين الفيروس تحـت الدراسـة BNRSV و بين أربعة عزلات من فيروس PNRSV فى هذا البحث .