Purification, serology and molecular detection of Egyptian isolates of banana bunchy top babuvirus and faba bean necrotic yellows nanovirus

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ABSTRACT

Two isolates of faba bean necrotic yellows nanovirus (FBNYV) and banana bunchy top babuvirus (BBTV) were purified. Cellulase added to the extraction buffers (EB) enhanced extraction of both viruses. An acidic pH was used for FBNYV extraction. Several trials for purification of BBTV indicated that alkaline condition surpassed acidic condition in virus extraction. Polyethylene glycol and NaCl were used to concentrate both viruses. Electroelution coupled with freezing and thawing were employed in further purification steps. Purified FBNYV yielded 677 μg/ g tissue and with A 260/280 ratio of 1.43. Purified BBTV yielded 12.9 μg /g tissue and with A 260/280 ratio of 1.3. Both viruses had a single-capsid protein of 20 kDa.

An antiserum raised for the intact virions of FBNYV detected the purified virus and was able to cross react with BBTV in dot blotting immunobinding assay (DBIA) and in western blotting (WB). Antiserum for the whole virions of BBTV detected the virus in its vector Pentalonia nigronervosa as well as in infected banana tissues. This antiserum also cross-reacted with FBNYV in DBIA and in WB but failed to react with its own antigen of BBTV. On the otherhand, an antiserum raised for the coat protein of BBTV was able to detect both FBNYV and BBTV in WB. Such results suggest the presence of continuous epitopes in FBNYV virions and discontinuous epitopes in BBTV virions.

Polymerase chain reaction (PCR) experiments indicated that primers for the coat protein and replicase genes of BBTV amplified products in FBNYV genome similar to those produced in BBTV genome. Similar results were obtained upon using immunocapture (IC) PCR (IC-PCR) technique; indicating strong serologic relationships between the two viruses. The use of specific BBTV primers for nanovirus detection is of a great value in sensitivity and suitable for large scale testing.

***Key words:** Purification, serology, molecular detection, BBTV, FBNYV.*

INTRODUCTION

*B*BTV is considered the most serious virus disease affecting banana worldwide. The disease was first recognized in Fiji in 1889, Taiwan in 1890, in Egypt 1901, and in Australia. 1913 (Fahmy, 1927; Wardlaw, 1961). It has spread and caused devastating problems in many countries including Fiji in 1927 and India and

Egypt in 1953, The Philippines and Taiwan in 1961, China in 1979 (Dale, 1987).

FBNYV caused economically important diseases of faba bean, lentil and pasture legumes in western Asia, north Africa, Sudan and Ethiopia (Makkouk *et al.*, 1992, 1994; Katul *et al.*, 1993; Shamloul *et al.*, 1999). Since the beginning of the 1990s this virus has been causing serious economic losses to faba bean in Egypt (Abdel-Salam and El-Sharkawy, 1996; Abdel-Salam, *et al.*, 1997).

Both *BBTV* and *FBNYV* are members of the family *Nanoviridae* which has two genera, viz. *Nanovirus* circumventing *FBNYV* and *Babuvirus* containing *BBTV* (Randels *et al.*, 2002). *FBNYV* shares vector-transmission (i.e. aphids) and particle properties, as well as genome composition and organization with *BBTV* (Harding *et al.*, 1991, 1993; Burns *et al.*, 1995; Franz *et al.*, 1998). *BBTV* is transmitted by the aphid vector *Pentalonia nigronervosa* in a semi-persistent manner and with no report of mechanical transmission. *BBTV* is also transmitted vegetatively through the suckers and tissue culture (Su, 1998). *FBNYV* is persistently transmitted by various aphids of which *Aphis craccivora* is the most significant natural vector (Franz *et al.*, 1998). Seed-transmission of an Egyptian isolate of *FBNYV* has been reported (Abdel-Salam and El-Sharkawy, 1996). Both *FBNYV* and *BBTV* have small isometric virus particles. *FBNYV* particles are 18 nm in diameter made up of a single capsid protein of about 22 kDa (Katul *et al.*, 1993) engulfing ten circular ssDNA (Katul *et al.*, 1998). Similarly, BBTV particles are 20 nm in diameter with a capsid protein of about 20-20.5 kDa and with a genome of six circular ssDNA (Burns, *et al.*, 1995; Su, 1995; Xie and Hu, 1995; Wanitchakorn *et al.*, 1997). In Egypt, El-Afifi (1985) found spherical particles with 23 nm in diameter in the ultrathin sections of *BBTV* banana infected leaves.

Currently, the recommended strategy for control of BBT disease is to detect virus-infected plants as early as possible, remove the diseased plants, and replace them with healthy ones (Dale, 1987). Such detection depends more accurately on serologic examination rather than visual observations. Serologic detection is hindered by difficulties in obtaining large amount of highly purified *BBTV* antigens. Similarly, *FBNYV* is difficult to purify for its being phloem limited mimicking *BBTV*. Generally, ultra-speed centrifugation used to purify both viruses may affect the purified virus yield due to shearing forces exerted on purified virions. Secondly, natural tendency of purified virions to aggregate with themselves or with plant cell membranes is present due to rich hydrophobic moieties present in both virions and plant membranes. The recent addition of cellulases and/or pectinases have helped in the purification of plant viruses such as *FBNYV* (Katul *et al.*, 1993; Abdel-Salam and El-Sharkawy, 1996), and banana streak badnavirus (Greeting *et al.*, 2000; Harper *et al.*, 2002).

Hafner *et al.* (1997) have demonstrated that the major gene of DNA-1 of *BBTV* encodes a replication initiation protein (Rep) while Wanitchakorn *et al.* (1997) have shown that DNA-3 encodes the viral coat protein.

The present paper is engaged with purifying both *BBTV* and *FBNYV* using low-speed centrifugation coupled with electro-elution techniques in an effort to overcome some of the obstacles resulting from high-speed centrifugation which lowered the virus yield. The study is also concerned with induction of good quality antisera for both viruses to be used in field detection as well as epidemiological studies. The obtainment of a good quality antiserum not only does help in reliable indexing assays for BBTV but also helps in its epidemiology (Xie and Hu, 1995).

Such induction of antisera to viruses with economic importance as BBTV and FBNYV using simplified purification techniques is invaluable especially for unequipped laboratories seeking cheaper technologies.

One of the main objectives of this study is also to test sensitivity of serological and molecular techniques for the detection of BBTV and FBNYV and to measure degrees of their relatedness. In doing so, polymerase chain reaction (PCR) and immunocapture (IC) PCR (IC-PCR) techniques have been employed. The development of IC-PCR by Wetzel *et al.* (1992), for instance, increased the sensitivity of PCR to about 5000 times than that of ELISA. Extracted information from such studies allow the development of more reliable techniques for their detection, and will also help in making decisions regarding the serological relationship between these two viruses.

MATERIALS AND METHODS

Virus sources

Two isolates of *BBTV* were used in this study. The first isolate was obtained from El-Menya governorate and designated as *BBTV-M*. The second one was obtained from El-Giza governorate (The Experimental Farm of the Faculty of Agriculture, Cairo University) and designated as *BBTV-G*. The two isolates were from banana plants showing typical bunchy top symptoms with congested leaves and narrow blades with yellow edges and typical Morse-code pattern on leaf petioles and stems. The *FBNYV* isolate was obtained from Assiut governorarte *(FBNYV-Ass)*. Infected plants showed leaf curling, yellowing and necrosis.

Insect transmission and virus propagation

Twenty tissue-cultured virus-free banana plants were used for the propagation of each isolate of *BBTV*. Ten adults of *Pentalonia nigronervosa* insects (collected from infested banana bunchy top-infected plants) were introduced to the tested banana plants using 72 hr acquisition-feeding period. Plants were kept during insect exposure under cages then sprayed with an insecticide. The inoculated plants were left for symptom development under greenhouse conditions and sprayed with an insecticide periodically. Virus-free *Aphis craccivora* insects raised on cabbage plants were transferred for 72 hr acquisition-feeding period on the obtained *FBNYV*-infected plants obtained from Assiut governorate. The aphids were then transferred onto seedlings of faba bean plants at the two leaf stage, kept caged, for 72 hr inoculation-access period. Ten viruliferous insects were used per plant. Plants were maintained under greenhouse conditions and sprayed against insects with an insecticide periodically.

Virus purification

FBNYV

The main frame for the purification of *FBNYV* is shown in Table (1). Sixty grams of freshly cut faba bean leaves showing symptoms of *FBNYV*-Ass infection were collected from the greenhouse after 28 days from exposure to viruliferous aphids. The leaves were minced (1:2.5, v/v) with a cold extraction buffer (EB) composed of 0.1 M sodium citrate buffer, pH 6.0 containing 20 mM Na_2SO_3, 0.1% (v/v) 2-mercaptoethanol, 0.1% (v/v) thioglycolic acid, and 0.1 % (w.v) cellulase (Sigma, C-7502) and left overnight at 4°C. The extract was filtered in a double layer of cheesecloth, shaken vigorously with 25% cold chloroform for 4 min, then centrifuged at 8000 rpm/10 min/5°C. The resulting supernatant was mixed with 10% (w/v) polyethylene glycol (PEG, mw. 8000) and 1% (w/v) NaCl, stirred for 30 min at room temperature (RT), then for two hr at 5°C. The

virus was pelleted with 10,000 rpm/10 min/4C, suspended (1/5 of the original vol. of the added EB) in a suspension buffer (SB) composed of 1 mM phosphate buffer, pH 7.4. The virus was electro-eluted in Blue Tank electrophoretic concentrator (Isco Inc., Lincoln, Nb, USA) using 20 mM phosphate buffer, pH 7.4, as a Tank buffer and 4 mA/cell. The eluted virus preparations were collected, suspended in SB using 1/50 vol. of the added EB, centrifuged 10,000 rpm/20 min/4C, then frozen at 17°C overnight. The purified virus was thawed, low-speed centrifuged again, then measured spectrophotometrically. Virus concentration was estimated using an $E^{0.1\%}_{260}$ of 3.6 (Chu and Helms, 1988).

BBTV

Tissue-cultured banana plants infected with *BBTV-G* under greenhouse conditions were used for the purification trials of BBTV. These trials had a common denominator of extracting the infected tissues with the tested buffer at a ratio of 1: 3 (w/v), concentrating the virus with PEG and NaCl, followed by electro-elution as previously followed in purifying *FBNYV*. However, different EB systems (with different pHs, ionic strengths, and additives), variable concentrations of PEG, NaCl, and SB were adopted in purifying *BBTV-G* as depicted in Table (2). In all procedures, except in procedure # 3, electo-elution of purified virus was made in 20 mM phosphate, pH 7.4, as a tank buffer. 1 mM phosphate, pH 7.4, was used as a suspension buffer. In procedure # 3, however, 60 mM sodium acetate, pH 6.0, was used as a tank buffer and 6 mM sodium acetate, pH 6.0, as a suspension buffer. Virus concentration was estimated assuming an $E^{0.1\%}_{260}$ of 3.6 (Chu and Helms, 1988). Purified virus preparations were treated afterwards as described in the purification procedure of *FBNYV* and preserved frozen.

Analysis of virus protein

Polyacrylamide gel electrophoresis (PAGE) was used for protein analysis under denaturing conditions. Suspensions of purified *FBNYV-A* and *BBTV-G* were each mixed with an equal volume of 2X dissociation buffer (DB), (0.15 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol) and heated up (90°C/1 min). Electrophoresis was in SDS-polyacrylamide gel (Laemmli, 1970). Gels (4% stacking and 10% resolving) were cast in a vertical mini-slab gel SE 280 apparatus (Hoefer-Sci. Inst., San Francisco, CA, USA). Electrophoresis was conducted at a constant 100 V for 3 hr using a Tank buffer composed of 25 mM Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS. Gels were fixed (30 min/ RT) in 25% isopropanol and 10% acetic acid, stained overnight/RT with rapid Commassie Brilliant Blue R staining solution (0.06%, w/v) prepared in 10% acetic acid, and destained in 7% acetic acid. Relative molecular weights (Mr) of the coat proteins were determined by linear regression analysis.

Serologic studies

Antiserum production

A polyclonal antiserum for *FBNYV* was raised through applying six weekly consecutive intramuscular injections of purified virus (3.6 mg/ml, each) into a Newzealand rabbit. In the first injection, the virus was emulsified with complete Freund's adjuvant. In the rest five injections, incomplete Freund's adjuvant was used. The rabbit was bled weekly for 8 weeks starting one week after the last injection. The obtained antiserum was designated as FBNYV-AS.

Two polyclonal antisera were raised for the *BBTV-G*. The first antiserum was obtained by injecting the rabbit with the intact purified virus (1.8 mg virus/injection), obtained from procedure # 5 following the above technique

described for the antiserum production of *FBNYV*. This antiserum for the whole (W) virus was designated as *BBTV*-AS (W). The second antiserum for BBTV was made by disrupting virions (DV) of 2.5 mg BBTV (procedure 5) with an equal volume of 2X DB. The preparation was heated up (100°C/ 3 min), cooled in ice, then dialyzed overnight at 4°C in 1 mM phosphate buffer, pH 7.4, containing 0.02 M Na_2SO_3 and 0.1% SDS. The dialyzed DV were divided into five equal aliquots each containing 0.85% (w/v) NaCl. Antiserum for *BBTV*-DV was obtained through five weekly consecutive injections into the rabbit after

described above for *FBNYV*. This antiserum was designated as BBTV-AS (DV).

The IgG fraction for each of the obtained antisera in this study was separated according to the technique described by (Mckinney and Parkinson, 1987).

The locally induced antisera and their respective IgG for either *FBNYV* or *BBTV* were cross absorbed with healthy tissues of faba bean and banana respectively as described by Abdel-salam (1999).

Serologic tests

1- Agar double diffusion test (ADDT):

ADDT was conducted to measure the antigenicity, immunogenicity, and serologic relationships between the purified *BBTV-G* and *FBNYV-Ass*. The gels composed of 0.8% (w/v), 0.85% NaCl (w/v), and 0.1% NaN_3 prepared in phosphate buffer saline, PBS, pH 7.4, (0.137 M NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.02% NaN_3).

2- Dot blotting immunobinding assay (DBIA):

DBIA was conducted as described by Abdel-Salam, (1999) using nitocellulose transfer membranes (0.2 m pore size, BioBind-NC, Whatman Int. Ltd., Modistone, England). The test was used to detect the presence of *FBNYV*, *BBTV-G* and M isolates in fresh leaves and purified preparations, and inside the insect vector transmitting BBTV. Further, DBIA was used to verify the nature of the viruses understudies upon using authentic antisera for *FBNYV* and cucumber mosaic virus *(CMV)*. For the authentication of *FBNYV-Ass*, a polyclonal antiserum for *FBNYV* was kindly presented by K. Makkouk, ICARDA, Aleppo, Syria). For the purity of the tested *BBTV* isolates, an antiserum for *CMV* (D. Puricfull, University of Florida, Gainesville, Fl, USA) was used. Unless otherwise stated, tested antigens (e.g. leaves, fruits) were diluted with STEP buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 8.3, containing 0.2 M of Na_2SO_3 and ethylene diamine tetracetate (EDTA), and 1.5 % Triton X-100 [TX-100]).

3- Western blotting (WB):

WB (Towbin *et al.*, 1979; Hammond, 1990) was performed to measure serologic relationships between FBNYV and *BBTV*. Polyvinyl difluoride membranes (PVDF, P-0807, Sigma) were used to transfer the proteins of FBNYV and BBTV from the gels obtained from SDS-PAGE. Protein transfer was performed using the fast semi-dry blotting apparatus (Biometra Fastblot, Germany). Transfer buffer (TB) composed of 25 mM Tris-HCl, 150 mM glycine, 10% methanol. For protein transfer, the PVDF membranes were wetted with 100% methanol for ten min then equilibrated with the TB for another 10 min prior to use. Electroblotting was run at 5 mA/cm^2 for 7.5 min. Transferred membranes were blocked with 1 mg/ml polyvinyl alcohol for 2 min then soaked in the primary antisera, 2 hr/RT. The membranes were washed three times (10 min, each) in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mm NaCl, 0.05 % Tween 20) then treated for two hr with the secondary antiserum (IgG, whole molecule, Sigma A3687) of goat antirabbit alkaline phosphatase (AP) conjugate. Membranes were washed three times in TBST then developed

using Naphthol-AS-MX phosphate/Fast Red or NBT/BCIP complexes as described by Abdel-Salam (1999).

Molecular studies

Nucleic acid extraction

Nucleic acids were extracted from the Egyptian isolates of *BBTV* and *FBNYV* purified virions as described by the technical bulletin of High pure PCR template preparation kit (Roche). 200 µl of purified virus was mixed with 200 µl of Binding buffer (6 M guanidine HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100(v/V), pH 4.4) and 40 µl of 20 mg/ml proteinase K was added. The mixture was incubated at 72°C for 10 minutes and 100 µl of isopropanol were added. The samples were pipetted into the upper reservoir of a combined High Pure filter tube-collection tube assembly and centrifuged in a standard table top centrifuge for 1 minute at 8000 rpm. The flowthrough was discarded and the filter tube was combined with a new collection tube. 500 µl of Inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6) was added to the upper reservoir and centrifuged for 1 min at 8000 rpm. The flowthrough was discarded again and 500 µl of wash buffer was added twice to the upper reservoir and centrifuged for 1 min at 800 rpm. The filter tube was inserted into a clean 1.5 ml reaction tube and 200 µl of a prewarmed elution buffer was added to the filter tube and centrifuged for 1 minute at 8000 rpm. The microfuge tube now contains the eluted DNA.

PCR amplification of BBTV and FBNYV

The coat protein gene (DNA-1), and the replicase gene (DNA-3) of both *BBTV* and *FBNYV* were generated by a PCR-based strategy using primers designed from the sequences of *BBTV* DNA-1, and DNA-3, (Harding *et al*., 1993; Burns *et al*., 1995) (Table 1). PCR mixes comprised 50 pmol of each primer, 10 mM dNTPs, 1 U *Taq* DNA polymerase (Roche) and 1 µl of total nucleic acid extract (diluted 1 to 10 in TE buffer, pH 8). The reaction mixes were denatured at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min followed by 1 cycle of 72°C for 10 min. Following amplification, PCR products were then electrophoresed onto 1% agarose gel and photographed using gel documentation system.

Table (1): Primers used in the amplification of both BBTV and FBNYV cp and rep genes.

Primer	Sequence (5' to 3')	Component	Location (nt)
BT1.1F	ATGACTTTACAGCGCACGCTCCGACAAAAG	DNA-1 (Rep)	970-999
BT1.1R	GTAATGGAGAGGGGGGAGGTCTATTTATAG		80-51
BT3.1F	TAAGCATCACACCCACCACTTTAG	DNA-3 (CP)	1016-1040
BT3.1R	GGGCCCTATATCCACAATCCATTA		72-49

IC-PCR

A polyclonal antibody against *FBNYV* was diluted 1:500 in coating buffer (pH 9.6) (100 mM $NaHCO_3$, 37 mM Na_2CO_3). Fifty microlitres of diluted antibody was incubated in a 0.5 ml PCR Eppendorf tube for 3 hr at 37°C. 100 ng of each of purified BBTV and FBNYV were mixed with 1x PBS pH 7.4; 2% PVP; 0.05% Tween 20. The tubes pretreated with the antibody were washed twice with washing buffer (1x PBS [pH 7.4], 0.05% Tween 20) and 100 µl of the purified viruses were added. The mixture was vortexed and centrifuged at 4000 xg for 7 min. The tubes were incubated at 4°C overnight and washed three times with washing buffer; 20 µl of the above BT1 F/R and BT3 F/R primer mix was added. PCR was performed using the above

cycle conditions. The amplification products from PCR were analysed on a 1% agarose gel stained in ethidium bromide and the bands were photographed under UV illumination.

RESULTS AND DISCUSSION

Virus purification

In the present study, several procedures were used for the purification of *FBNYV* and *BBTV* isolates. These procedures were based originally on extracting the sap containing virus at either acidic pH for *FBNYV* and/or acidic, neutral, and alkaline pHs for *BBTV*. Various additives (e.g. Na_2SO_3, 2-ME, TGA) were added to the EB to reduce the actions of polyphenol oxidizing enzymes. Digestive enzymes (e.g. cellulase, pectinase) were also included in the EB to facilitate virus extraction. Both viruses are mostly phloem limited due to their nature of persistent-aphid transmission. The clarification step for both viruses depended on using chloroform as a clarifying agent alone or with butanol. PEG and NaCl followed by electroelution (EE) and low-speed centrifugation steps replaced the differential centrifugation and/or sucrose density gradient steps used to purify these two viruses (Katul *et al.*, 1993; Xie and Hu, 1995; Sadik *et al.*, 1999). Purified *FBNYV-ASS* had A max at 260 $_{nm}$, A $_{min}$ at 240, and A 260/280 ratio of 1.43. These results are typical to those reported by Katul *et al.* (1993) and Brunt *et al.* (1996). Yield of the purified virus was 677 g/g tissue (Table 2 and Fig. 1). Such yield to our surprise was much higher than comparative yield (1mg/kg of pea tissues) reported by Katul *et al.* (1993). This could be attributed to: a) extracting the virus from its original host (i.e broad bean), b) the use of cellulase in EB instead of pectinase reported by Katul *et al.* (1993), and c) the use of low speed centrifugation and EE to concentrate and purify the virus instead of ultra centrifugation (Katul *et al.*, 1993) where a great deal of virus is lost.

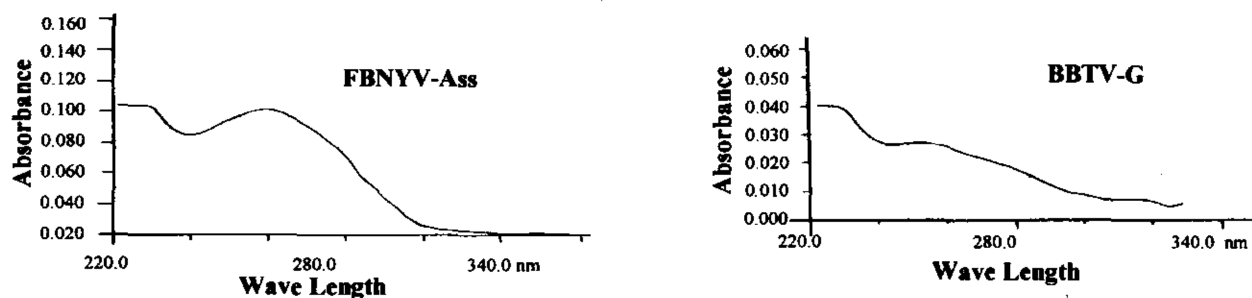

Fig. (1): Ultraviolet spectra of purified FBNYV-Ass and BBTV-G.

All the used purification methods for *BBTV-G* (Table 2, Fig.1) had A 260/280 ratio of 1.3, except for method # 3, which gave an A 260/280 ratio of 1.2. Such 1.3 ratio agrees with other results reported for *BBTV* by Thomas and Dietzgen (1991) and Sadek *et al.* (1999). Results in Table (1) indicated that methods #5 (pH 7.4) followed by # 4 (pH 7.0) had surpassed all the other methods (pH 6.0) in virus yield. It seems that acidic pH medium

is not preferable for *BBTV* extraction when compared with similar extraction media where neutral and alkaline pHs are used. Yield of purified BBTV in method 5 (12.9 µg/g tissue) was higher than yields reported by Iskra *et al.* (1989) and Sadik *et al.* (1999). The EB in method 5 was somehow similar in its structure to the one described by Sadik *et al.* (1999) except for the addition of cellulase. Similarly, in method 5, the virus was concentrated by adding polyethylene glycol and NaCl. However, no EE steps were followed to further purify the virus in the technique of Sadik *et al.* (1999). This perhaps explains the benefits of both adding cellulase to the EB and concentrating the virus with EE in enhancing the virus yield and evading the shearing forces exerted on the purified virions through ultra centrifugation. The use of EE in virus purification has proven successful in purifying *cotton leaf curl mosaic begomovirus* (Abdel-Salam, 1999) *tobacco ringspot nepovirus* (Abdel-Salam *et al.*, 2003) as well as other tricho- and potyviruses (Abdel-Salam, unpublished).

Table (2): Various procedures used in purifying the local isolates of FBNYV-Ass and BBTV-G.

Method #	Extraction buffers	pH	Clarifying Agents	Concentra-ting Agents	A 260/280 Ratio	Virus Yield µg/g tissue
FBNYV-Ass						
1	0.1 M sodium citrate, 20 mM Na_2SO_3, 0.1 % 2-ME, 0.1 % TGA,0.1 % cellulase	6.0	25% $ChCl_3$	10% PEG + 1%NaCl	1.4	677
BBTV-G						
1	0.1 M sodium Citrate, 20 mM Na_2SO_3, 0.1 % TGA, 0.1%2-ME, 0.1% cellulase	6.0	25% $ChCl_3$	10% PEG + 1%NaCl	1.3	4.4
2	0.1 M sodium Citrate, 20 mM Na_2SO_3, 0.1 % TGA, 0.1%2-ME, 0.1% cellulase, 0.1% pectinase	6.0	-	8%PEG + 1%NaCl	1.3	2.6
3	0.5 M sodium acetate, 0.1% DIECA,.5% sucrose	6.0	25% $ChCl_3$	8%PEG + 2%NaCl	1.2	3.4
4	10 mM sodium phosphate, 20 mM Na_2SO_3, 0.1 % TGA, 0.1%2-ME, 0.1% cellulase	7.0	25% $ChCl_3$	10% PEG + 1%NaCl 0.277g/ml ammonium sulfate	1.3	8.7
5	0.1 M potassium phosphate, 20 mM Na_2SO_3, 0.1%TGA, 0.1% cellulase	7.4	12.5%$ChCl_3$12.5% butanol	8%PEG + 2%NaCl	1.3	12.9

Abbreviations: A = Absorbance, DIECA = sodium diethyldithicarbamate , 2-Me = 2-mercapto ethanol, TGA =

Analysis of virus coat protein

Both proteins of FBNYV and BBTV-G migrated as single components simultaneously in SDS-PAGE under denaturing conditions. Estimated coat protein Mr for both viruses was 20 kDa (Fig. 2). Such results are similar to those reported for other nanoviruses (Brunt *et al.*, 1996). Katul *et al.* (1993) reported, however, a value of 22 kDa for the coat protein of FBNYV. Values of 20.1-20.5 kDa for the coat protein of BBTV have also been reported (Xie and Hu ,1995; Wanitchakorn *et al.*, 1997; Su ,1998).

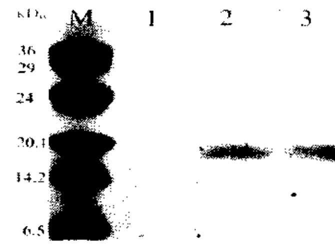

***Fig. (2):** SDS-PAGE showing similarities in coat protein subunit molecular weigh of FBNYV-Ass and BBTV-G. M, molecular weight Markers (Sigma) in kDa including aprotinin (6.5), lactalbumin (14,2), Trypsin inhibitor (20.1), Trypsinogen (24), carbonic anhydrase (29), glyceraldhyde-3-phosphate dehydrogenase (36). Lane 1, Blank: lane 2. FBNYV-Ass: Lane 3. BBTV-G.*

Serologic studies

1- Agar double diffusion test (ADDT):

BBTV-AS(W) did not react with purified *BBTV* or *FBNYV* antigens (Fig 3-A). *FBNYV*-AS, on the otherhand, reacted with its own purified antigen but not with purified BBTV.

2- Dot blotting immunobinding assay (DBIA)

CMV(AS):

Both isolates of *BBTV-G* and *M* failed to react with *CMV*-As (Fig. 4, panel I); indication of the purity of both virus isolates from any *CMV* contaminant.

FBNYV(AS):

Purified *BBTV-G* reacted poorly and moderately with *FBNYV*-AS (Makkouk) and to the locally induced *FBNYV*-AS (Fig. 4, panels II and III, respectively). On the otherhand, purified *FBNYV-ASS* reacted strongly with its own two tested antisera for FBNYV (Fig. 4, panels II, and III, respectively). No reactions were observed with healthy controls (Fig. 4).

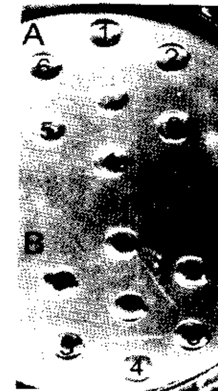

***Fig. (3):** Agar double diffusion test showing the reaction of: BBTV and FBNYV antisera with their corresponding anitgens. Central wells, (A) BBTV-AS (W) and (B) FBNYV-AS, each diluted 1/3 with PBS. Peripheral wells in both (A) and (B) are as follow: # 1, 3, 50 g/well of purified BBTV-G (intact virus); 2, 4, 50µg/well of purified FBNYV; 5, 6, healthy sap, diluted 1/3 with PBS, of banana and faba bean leaves, respectively.*

BBTV(AS):

Virions of purified *FBNYV-Ass* and *BBTV-G* reacted with *BBTV*-AS (W), (Fig. 5; lanes 1,2, respectively). *BBTV*-AS (W) detected *BBTV-M* in extracts of viruliferous *P. nigronervosa* insects as well as in extracts of tissue cultured banana plants exposed to viruliferous *P. nigronervosa* insects (Fig. 5; lanes 3,4, respectively). No positive reactions were detected upon testing *BBTV*-AS (W) with extracts from healthy banana leaves or fruits (Fig. 5; lanes 4, 5, respectively).

3- Western blotting:

Results in Fig. (6-A) indicated that *FBNYV*-AS was able to detect the coat protein antigen of *FBNYV-Ass* as well as, but poorly, the coat protein antigen of *BBTV-G*. Antiserum for *BBTV-G* prepared for the whole virus [*BBTV*-AS(W)] failed to recognize its own *BBTV-G* antigen, but reacted with *FBNYV* antigen (Fig. 6-B). On the otherhand, *BBTV* antiserum prepared from disrupted viroins [*BBTV*-AS(DV)] reacted with its own coat protein antigen as well as *FBNYV* coat protein antigen (Fig. 6-C).

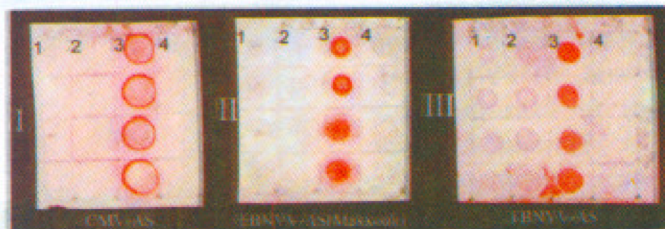

Fig. (4): DBIA showing the reaction of BBTV-G, M isolates and/or FBNYV-Ass isolate with CMV and FBNYV antisera. Panel I, reactivity of CMV (diluted 1/250 with TBST) with infected banana leaves with 1 (BBTV-G) and 2 (BBTV-M), 3 (positive control of CMV-infected banana), and 4 (healthy banana). Panel II, reactivity of FBNYV-AS (Makkouk), (diluted 1/1000 with TBST) with 1, 2 (purified BBTV-G, 1mg/ml,), 3 (purified FBNYV-Ass, 1 mg/ml), and 4 (healthy faba bean). Panel III, reactivity of the induced antiserum for FBNYV-Ass (diluted 1/1000 with TBST) against 1, 2 (purified BBTV-G, Img/ml), 3 (purified FBNYV-Ass, 1 mg/ml), and 4 (healthy faba bean). Each tested antigen was applied as 1 l per square marked on the membrane. Goat antirabbit alkaline phosphatase conjugate was used at a dilution of 1/1000. Nitrocellulose membranes were stained with Naphthol/Fast red complex). In each panel, treatments were repeated four times.

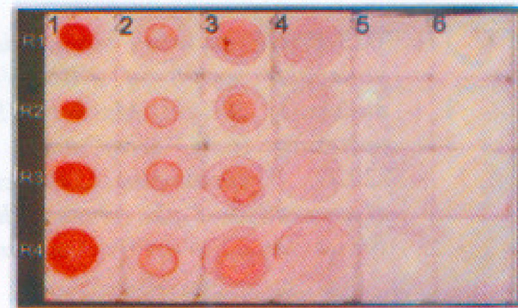

Fig. (5): DBIA showing the reactivity of BBTV-AS(W), diluted 1/250 with TBST, with 1 (purified FBNYV-Ass, 7.5 mg/ml), 2 (purified BBTV-G, 7.5 mg/ml), 3 (extracts of viruliferous Pentalonia nigronervosa, 10 insects/30 µl TBST), 4 (diseased tissue-cultured banana exposed to virulierous P. nigronervosa carrying BBTV-M, 5 (healthy banana leaves), and 6 (healthy banana fruits). Each tested antigen was applied as 1µl per square marked on the membrane. Goat antirabbit alkaline phosphatase conjugate was used at a dilution of 1/2000. Blots were stained with Naphthol/Fast red complex). Each treatment was repeated four times (R1 R4).

The results in Fig. 6 can be explained basically on the presence of two types of conformational epitopes, i.e. continuous and discontinuous epitopes (Mernaugh *et al.*, 1993). *FBNYV*-As reacted with the intact and disrupted *FBNYV* virions in DBIA and in WB, respectively, due to the presence of continuous epitopes that can be serologically active either in the nature form of the virions or upon denaturation with SDS. On the otherhand, The positive reactions of BBTV-AS (W) with the intact virions of *BBTV* and *FBNYV* in DBIA (Fig. 5) and only with *FBNYV* in WB (Fig. 6-B) are indications of the presence of

continuous epitopes in *FBNYV* and discontinuous epitopes (cryptopes=hidden epitopes) in *BBTV*. This conclusion was further confirmed when *BBTV*-AS (DV), originally prepared for the coat protein of *BBTV* was able to react in WB with both disrupted virions of *FBNYV* and BBTV (Fig. 6-C).

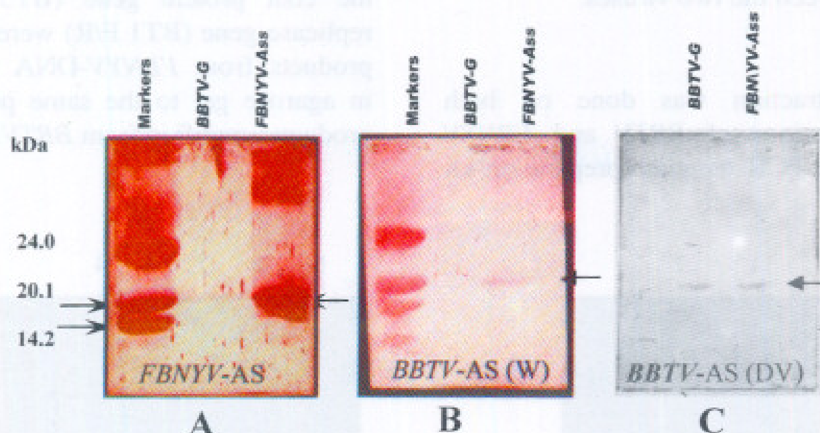

Fig. (6): Western blotting (WB) tests showing the reactions of dissociated purified antigens of FBNYV and BBTV with their corresponding antisera. The tested three antisera (using the IgG fractions) were used at a dilution of 1/10 (100 μg g/ml). Purified FBNYV and BBTV run in SDS-PAGE before electroblotting were used at 3.7 and 2.1 mg/ml, respectively. In A, electroblotted antigens were examined for FBNYV-AS then stained with Naphthol/Fast Red. To prepare blotted membranes in B and C purified antigens of FBNYV and BBTV were repeated twice in the gel, run, and the gel was halved where each half contained a copy of the same antigens. In B, the gel was blotted on PVDF membrane, treated with BBTV-AS(W) and finally stained with Naphthol/Fast Red; while in C, the same co-electrophoresed antigens were blotted onto PVDF membrane, treated with BBTV-AS(DV), then finally stained with NBC/BCIP. In all three membranes, the secondary antiserum-conjugated AP and the streptavidin-AP conjugate were used at dilutions of 1/5000, and 1/1000, respectively. Markers used in WB were biotinylated according to Hill (1993). Arrows on the right of each membrane depict the position of the blotted peptides at 20 kDa.

Similarities in the coat protein Mr, migration in PAGE, serologic cross reactivities of antisera of both viruses with their respective antigens indicate close chemical and serologic relatedness between the two viruses and agree with results of several authors (Harding *et al.*, 1991, 1993; Burns *et al.*, 1995; Franz *et al.*, 1998). However, other results indicated no serologic relatedness between the two viruses based on ELISA and immunoelectron microscopy (IEM) results (Katul *et al.*, 1993). Further Katul *et al.*, (1998) indicated the presence of less than 24.6 % identities between *BBTV* and *FBNYV*. However, such negative

results in ELISA and IEM might be attributed to the sensitivity of the tests. For instance ADDT (Fig. 3) failed to measure cross-reactions between the two viruses. However, DBIA and WB, more sensitive and accurate tests than ELISA, were able to measure close similarities between the two viruses.

DNA-PCR

DNA extraction was done on both purified preparations of *BBTV* and *FBNYV* using High pure PCR template preparation kit (Roche) which yielded DNA suitable for PCR in only 1 hour. The supplied PCR mix improved PCR performance considerably by yielding intense positive signals with only 1l of template in PCR (Fig. 7 and 8). Results in Figs. 7 and 8 showed that *BBTV* primers for the coat protein gene (BT3 F/R) and the replicase gene (BT1 F/R) were able to amplify products from *FBNYV*-DNA which migrated in agarose gel to the same position of those products amplified from *BBTV*.

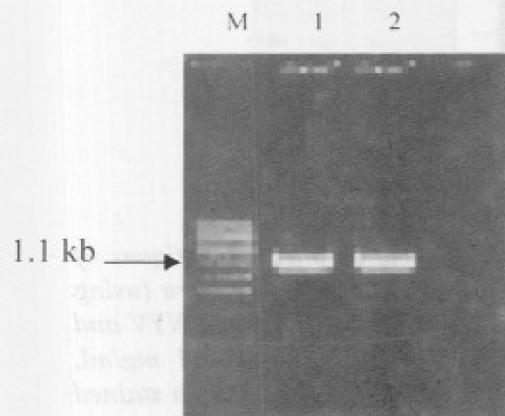

Fig. (7): 1% agarose gel electrophoresis of PCR products derived from purified BBTV. Lanes 1 and 2: Amplification products using primers specific for BBTV coat protein and replicase genes, respectively. Lane 3: No PCR products were produced from DNA extracted from healthy banana and amplified with primers specific for BBTV coat protein gene. M: Molecular weight markers (Roche).

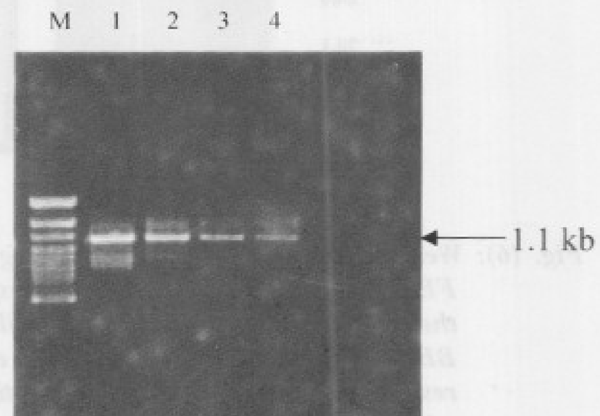

Fig. (8): PCR products amplified from BBTV and FBNYV DNA molecules extracted from purified virions. Lanes 1 and 2: PCR products of CP and replicase genes amplified from BBTV (Lanes 1 and 2) and FBNYV (Lanes 3 and 4). All amplifications were performed by using primers specific for the CP and replicase genes of BBTV. M: Molecular weight DNA marker (Roche). The arrow points to the correct size of both amplified PCR products.

IC-PCR

One µl of the viral DNAs yielded amplification products comparable in intensity to 100 ng of purified virion preparations from *BBTV* and *FBNYV* in IC-PCR (Figure 9). The IC-PCR products of the coat protein genes of *BBTV* (Lane 1) and *FBNYV* (lane 2) appeared as a 1.1 kb band in each virus. The IC-PCR products of the replicase genes of BBTV (lane 3) and that of *FBNYV* (lane 4) appeared each as a 1.1 kb band. This indicates

strong serologic relatedness between BBTV and FBNYV.

An IC-PCR test method for *BBTV* and *FBNYV* were established based on an immunocapture step combined with PCR applying BT1 F/R and, BT3 F/R primers on both sequences. This assay is very sensitive and detects both viruses. The IC-PCR test method is rather inexpensive.

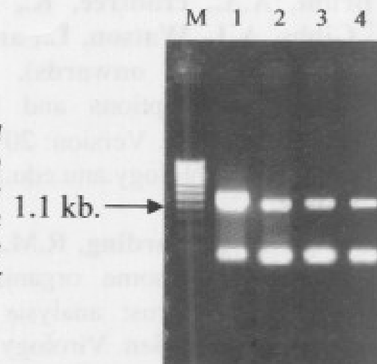

Fig. (9): Agarose gel electrophoresis of IC-PCR products of BBTV and FBNYV using primers specific for the coat protein (Lanes 1 and 2) and replicase genes (Lanes 3 and 4). The IC-PCR products of the CP genes of BBTV (Lane 1) and FBNYV (Lane 2) appeared as a 1.1 kb band in each virus. The IC-PCR products of the replicase genes of BBTV (Lane 3) and FBNYV (Lane 4) exhibited similar bands of the same size (1.1 Kb).

The comparison of the techniques revealed a very strong relationship between serological (Western blotting, DBIA) and molecular (PCR, IC-PCR) methods for the detection of *BBTV* and *FBNYV*. The reliability of the tests was mainly dependent on leaf sampling, sensitivity and specificity of the antisera for serological methods and the primer specificity for PCR techniques. The preferred method is IC-PCR test because it is highly sensitive for the detection of RNA and DNA viruses, rather than ELISA. IC-PCR proved to be more sensitive and reliable than DAS-ELISA (Wetzel *et al.*, 1992; Maria *et al.*, 2001). When compared to ELISA tests or DNA extraction procedures and subsequent PCR detection, IC-PCR, for its simplicity, sensitivity (reducing the risk of false positive results) and reproducibility, had some advantage. Therefore, IC-PCR can be regarded as a valuable alternative for large scale testing of nanovirus. The major disadvantage is that antisera are not always available for all known nanovirus. However, tests for the detection of *BBTV* and *FBNYV* were carried out on both viruses by using specific primers for *BBTV* coat protein and replicase genes. Therefore, further work is needed to detect *FBNYV* coat protein and replicase genes by using *FBNYV* specific primers.

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الملخص العربي

التنقية والكشف بالطرق السيرولوجية والجزيئية على فيروسي تورد القمة في الموز واصفرار وموت الفول البلدي

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تم فصل وتنقية عزلتان من فيروس اصفرار وموت الفول البلدي Faba bean necrotic yellows virus (FBNYV) وفيروس تورد القمة في الموز Banana bunchy top virus (BBTV) ووجد أن إضافة السليوليز لمحلول الاستخلاص يؤدى إلى زيادة محصول الفيروس المنقى لكلا الفيروسين. ووجد أن الوسط الحمضي يلائم استخلاص FBNYV ؛ بينما يلائم الوسط القلوي استخلاص BBTV وقد استخدم البولي اثيلين جليكول و كلوريد الصوديوم لتركيز الفيروسين تحت الدراسة. واستخدمت تقنيات الإزاحة الكهربية والتجميد والإذابة لزيادة نقاوة الفيروسين. وكان تركيز FBNYV يساوى ٦٧٧ ميكروجرام/جرام نسيج، وذو نسبة امتصاص عند أطوال موجية ٢٦٠/٢٨٠ نانوميتر تساوى ١,٤٣ . بينما أعطت دراسات مقارنة ل BBTV قيمة تساوى ١٢,٩ ميكروجرام/جرام نسيج، ونسبة امتصاص تبلغ ١,٣. ووجد أن الغلاف البروتينى لكلا الفيروسين يتكون من بروتين مفرد وذو وحدات بناء تبلغ ٢٠ كيلو دالتون للوحدة .

وبينت النتائج أن المصل المنتج للفيروس الكامل ل FBNYV يتفاعل مع انيجينة المنقى وكذا مع BBTV عند استخدام اختبار الطبع بالتنقيط(Dot blotting immunobinding assay (DBIA ،وكذا اختبار Western blotting (WB) وبينت الدراسة أيضا أن المصل المنتج للفيروس الكامل ل BBTV يستطيع الكشف عنBBTV في الحشرة *الناقلة Pentalonia nigronervosa* وفى أنسجة الموز المصابة؛ كما يتفاعل مع FBNYV عند استخدام اختباري DBIA و WB . إلا أن هذا المصل فشل في التفاعل مع انتيجينه في اختبار WB . وعلى الجانب الآخر، وجد أن المصل المنتج للغلاف البروتيني ل BBTV يستطيع الكشف عن انتيجينات BBTV و FBNYV في اختباري DBIA و WB. وتقترح النتائج المتحصل عليها تواجد مواقع انتيجينية antigenic sites = epitopes من النوع المتصلcontinuous في جزيئات FBNYV . أما في حالة BBTV، فأغلبها من النوع غير المتصل discontinuous epitopes.

وأوضحت نتائج اختبار تفاعل البلمرة المتسلسل Polymerse chain reaction (PCR) أن البادئات المتخصصة لجين الغلاف البروتيني وجين الربليكيز لفيروس BBTV تستطيع أن تبلمر نواتج متطابقة لكلا الفيروسين. وتم تأكيد تلك النتائج بنتائج مقارنة Immunocapture (IC) PCR والذي استعمل فيه مصل FBNYV والتي بينت القرابة السيرولوجية بينهما . وتوضح تلك النتائج إمكانية استخدام البادئات الخاصة ب BBTV في الكشف عن FBNYV على مدى واسع وبدقة عالية.