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COMPARISON OF PURIFICATION METHODS EMPLOYED FOR PAKISTANI ISOLATE OF BANANA BUNCHY TOP VIRUS

TAHIRA YASMIN, HUSSAIN SHAH AND SAIF KHALID

Plant Virology Lab., Crop Diseases Research Program,
National Agriculture Research Centre, Park Road, Islamabad, Pakistan

Abstract

Banana Bunchy Top Virus (BBTV) particles were purified employing various purification methods. Substantial amount of virus particles were obtained using tangential flow filters of millipore sizes of 10 and 0.16µm and 100KDa/100mwco sequentially. The method was most successful and gave high virus yield (75µg/ml) devoid of most host macromolecules. Under electron microscopy (EM) isometric virus particles of 18-20 nm in diameter were observed. The purified virus was used to raise polyclonal antiserum against local isolate of BBTV giving an antibody titre of 1:6000 higher than other methods. Later Enzyme-linked immunosorbent assay (ELISA) was also standardized for BBTV indexing.

Introduction

Banana (*Musa* spp.) is cultivated throughout the warmer countries confining to regions between 30° N and 30° S of the equator (Memon, 1996). Banana comes fourth after rice, wheat and maize with regard to gross value on the global scale. In Pakistan banana enjoys a favorable position among fruits as well as an important export commodity. Sindh is the leading banana growing province occupying 87% of the area under cultivation.

Among various pathogenic diseases two viruses have been reported and identified in banana crop i.e. Banana Bunchy Top Virus (BBTV) in 1992 and Cucumber Mosaic Virus (CMV) in 1998, (Khalid & Soomro, 1993, Khalid *et al*., 1999). The ravages brought by BBTV has wiped out banana crop from a few traditional banana-cultivating areas of Sindh since its emergence in epidemic form in 1991-92 with a loss of Rs. 915 million and 30% yield reduction within two years (Bashir & Hassan, 1998, Yasmin, 2001). Proper disease diagnosis is a pre-requisite for timely management of the pathogen. Serology is generally employed for the exact detection of the viral entity. Difficulties are generally encountered during virus purification for production of quality antiserum. To overcome such problems (banana contains a large amount of latex, tannin and phenolic compounds which largely interfere with the purification protocols), the present study was designed to explore an efficient and successful method of virus purification free from host contaminants to produce high quality antisera. In addition, purified virus can also be used for further characterization and biochemical analysis.

Materials and Methods

Purification of BBTV: Naturally BBTV-infected plants of *Dwarf Cavendish* and *William Hybrid* varieties collected from district Thatta were used for all purification methods. The source plants showing characteristic disease symptoms were further confirmed by serology (ELISA). The following three purification methods were used:

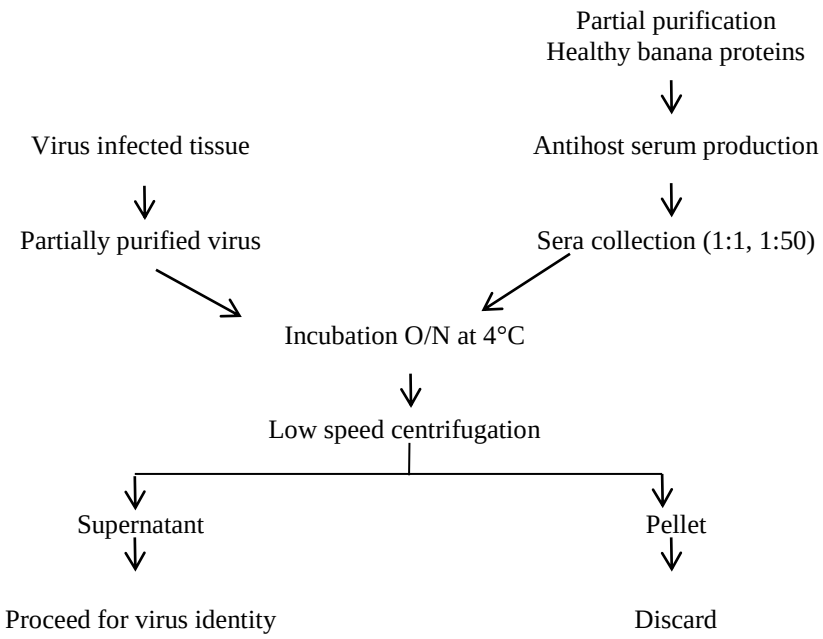

Fig. 1. Scheme of virus purification following the method of Gold (1960) and Van Regenmortel (1964).

Method I: Host proteins were first extracted from healthy banana and these were used for antihost serum production following the combined methods of Gold (1961), Fulton (1967) and Van Regenmortel (1964). BBTV was partially purified and incubated with antihost serum (diluted 1:1 and 1:50) at 4°C overnight to remove maximum host proteins from virus preparation. Final preparation was observed under EM. O.D of final virus preparation was determined by spectrophotometer at 260nm (Fig. 1)

Method II: Virus purification was done using conventional procedure following the method of Wu & Su (1990) with some minor modifications. Young leaves and pseudostems showing dark green streaks collected from rosetted plants, were pulverized in liquid nitrogen and homogenized with 0.02 M KPO_4 buffer containing 0.2% (v/v) 2-mercapto-ethanol and 0.1% (w/v) sodium diethyldithiocarbamate (Na-DIECA) and stirred for 30 minutes at 4°C. The mixture was emulsified with n-butanol/chloroform 10% (v/v), stirred for one hour and subjected to two cycles of differential centrifugation (36,000rpm for 2.5 hrs and 7,000rpm for 10 min). Finally the partially purified virus preparation was layered on 10-40% sucrose density gradient and centrifuged at 26,000 rpm for 3.5 hours. The zone containing virus particles was located and collected by measuring absorbance through spectrophotometer. The preparation was fractionated of 2 ml each and Optical density (O. D) was measured at 260nm. Fractions having absorbance values above 0.6 were used further for equilibrium centrifugation in Cesium sulfate (Cs_2SO_4) solution and virus band was finally resuspended in 1ml buffer. The integrity and stability of purified virus preparation were further confirmed by EM.

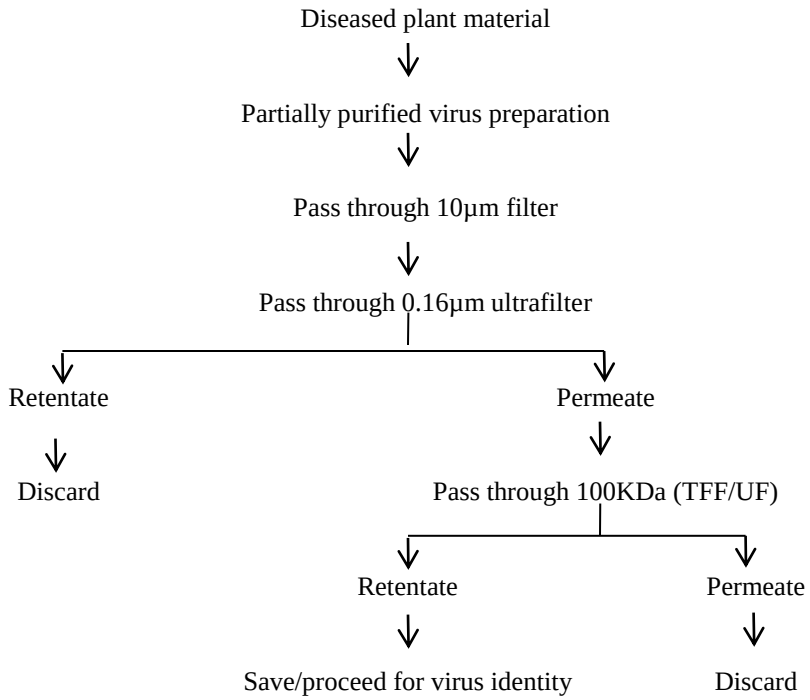

Fig. 2. Schematic diagram of virus purification through ultra-filtration.

Electron Microscopy (EM): Carbon coated Copper grids (300 mesh) were floated on a drop (20µl) of virus suspensions for 1 minute, rinsed with distilled water and stained with 2% Uranyl acetate (UA) pH=4.8 or 1% Phosphotungstic acid (PTA) pH= 6.5, for 30 seconds, filter dried and observed under EM. Later for size determination, particles were measured with fine divider from electron micrographs of known magnification and enlargement. About 100-200 virus particles were measured in cm/mm and the data converted into nanometer. Group categories were plotted on X-axis while number of size was plotted against each category on vertical axis in the form of a histogram and mean modal diameter was determined.

Method III: Initially virus particles were partially purified following the method of Wu & Su (1990). After clarification of mixture (260ml), it was first passed through 10µm pore size Polypropylene filter (Pall Corp) placed in polycarbonate housing (Pall. USA) and later through tangential flow filter (TFF) or ultrafilter (UF) having pore size of 0.16µm (160nm) and 100Kda or 100mwco using peristaltic pump with a variable flow rate (Fig. 2 & 3). All retentate and permeate samples were taken at each stage of the process and tested for virus presence through EM and spectrophotometry. Final retentate of 2nd Ultra-filtration was centrifuged at low speed (10,000rpm, 10min) and supernatant was collected. Samples collected from each ultra-filtration (retentate/permeate) were observed under EM and O.D was also determined at wavelength of 260nm.

Polyclonal Antiserum Production: Purified virus preparations obtained from method II and III, were used as immunogens for the production of polyclonal antiserum against BBTV. Purified virus preparation quantified (adjusted to 100-150µg/ml), emulsified with an equal amount of Freund's complete adjuvant and injected intramuscularly into chinchilla rabbit at three multiple sites. Later 3-4 injections were administered at 10 days interval by emulsifying the virus preparation with an equal amount of Freund's incomplete adjuvant. After 50 days, blood (6-8ml) was taken and serum separated. Booster injections were administered and antibodies titre was checked finally. Polyclonal antiserum as well as IgG (γ-globulin) titre and concentration was determined by agar gel diffusion test (AGDT) and DAC-ELISA.

To remove the host protein, healthy banana sap was extracted (1:10w/v) and 100ml of acetone was added to it. Tissue was separated from the acetone and the process was repeated over three times. Finally antiserum was incubated with the healthy sap for 1-2 hours at 35ºC and used in ELISA. BBTV antibodies (IgG fraction) were separated from serum through Ammonium sulfate precipitation. Polyclonal antiserum was diluted 10 times with distilled water. Diluted antiserum was added to 4 M Ammonium sulfate solution (1:1 v/v) drop-wise with constant stirring to precipitate immunoglobulins. The mixture was incubated for an hour at 4°C. The precipitates were collected by centrifugation at 10,000rpm for 10 minutes and dissolved in 0.5M Phosphate Buffer Saline (PBS). The suspension was further dialyzed against PBS (pH=7.8) and concentrated at low speed. Titre of purified IgG was confirmed through DAC-ELISA. Extinction coefficient of purified IgG was determined by measuring absorbance at 280nm. For the determination of antiserum titre following techniques were employed:

Agar gel diffusion test (AGDT): Pure agar (7.5 g), 9 g of NaCl and 0.4 g of $NaNO_3$ were dissolved in one litre of distilled water. Molten agar was poured into Petri dishes to a depth of 5mm (5-15 ml), allowed to solidify and equi-distance wells were cut using a template. Antiserum was diluted 1:100, 1:500, 1:1000, 1:5000 and 1:10000 while antigen was diluted tenfold. Antigen (20 µl) was added in the central well while antiserum dilutions (20 µl) in the peripheral wells. Agar plates were incubated at room temperature overnight in a humid chamber. Precipitation lines were observed against a light source.

Direct Antigen Coating (DAC) ELISA: Healthy and infected banana tissues were extracted in carbon coating buffer (0.05M carbonate buffer). The sap extract was centrifuged at 10,000 rpm for 5 minutes, supernatant was collected and diluted 1:10, 1:20 and 1:100. Polystyrene ELISA plates were coated 100 µl/well with antigen along with positive and negative controls and incubated overnight at 4°C. Plates were washed three times with 3 minutes interval in washing buffer (PBS-Tween 20). Antiserum dilutions (see Table 1) were loaded 100 µl/well and incubated at 37°C for 2-3 hours. Plates were again washed. Whole molecule conjugate (anti rabbit IgG, Sigma brand) was used at 1:500, 1:1000, 1:2000 and 1:4000 dilutions in PBS-Tween-PVP, added 100µl/well and incubated and washed as before. Substrate (p-nitrophenyl phosphate) @ of 0.6 mg/ml dissolved in substrate buffer (10% Diethanolamine, pH=9.8) was added @ 150 µl/well. Results were rated visually (- = no reaction, + = mild reaction, ++ = moderate reaction and +++ = severe/heavy) for one hour at room temperature and later absorbance was determined spectrophotometrically at 405nm.

Table 1. Comparison of BBTV purification methods.

Parameters	Method II	Method III
Particles observed under EM	Few	Many
Absorbance at 260nm	0.78-1.0	0.8-1.5
Purity	More host macromolecules	Less
Behavior under EM	Aggregated	Non-aggregated/scattered
Yield	180µg/Kg	750µg/Kg
Stability	Mostly broken	Complete
Economical	Cumbersome, lengthy & more analytical	Easy, short with single analytical step

Standardization of ELISA: One form of direct and two forms of indirect ELISA, described earlier, were standardized. All tissues were used fresh each time. Double antibody sandwich (DAS-ELISA) was performed as described by Clark & Adams (1977). Similarly triple antibody sandwich ELISA (TAS-ELISA) was employed as reported by Dietzgen & Thomas (1991) with some minor changes.

Results

Method I: This method of virus purification was not successful and did not yield virions free from host macromolecules. Both dilutions of antiserum 1:1 and 1:50 did not adsorb host proteins from partially purified virus preparation. Few isometric BBTV particles were observed under EM in final virus preparation, giving low absorbance values at 260nm (0.36, 0.40) with lowest virus yield of 3.5µg/ml or 35µg/Kg using freshly harvested infected banana tissue.

Method II: The method of Wu & Su (1990) with minor modifications was found partially successful as uniform isometric BBTV particles were seen under electron microscope. Freshly harvested infected tissue preparation gave more virus yield and contained high virus concentration as compared to recovery from frozen material stored at -70°C. A large number of virus particles were observed after first ultracentrifugation but were lost during successive centrifugation. Few virus particles were obtained after differential and density gradient centrifugation but not found free of host contaminants. However, impurities in final preparation were comparatively less after density gradient centrifugation. No virus particles were obtained in preparations made from healthy banana leaves processed identically.

From the fractions of sucrose density gradient centrifugation, fraction 7, 8 and 9 gave highest absorbance values of 0.87, 0.89 and 1.80, respectively. These fractions contained numerous isometric virus particles of uniform shape and size as compared to others when examined under EM. Scattered BBTV particles were observed in these fractions without host contaminants as compared to preparation of first and second ultracentrifugation. However, the preparation from density gradient centrifugation was impure. Uniform particles were observed along with host plant contaminants, measuring 18-20nm in diam., (Fig. 4). Virus particles were also observed in fraction 7, 8 and 9 obtained from sucrose and cesium density gradient centrifugation with no virus particles in fractions from control, despite numerous attempts. For staining purpose, 2% UA gave good contrast as compared to PTA. For size determination, the histogram represents the diameter distribution of BBTV particles measured from electron micrographs (Fig. 5), which shows the maximum number of particles with modal diameter between 19 to 21nm. Following the purification method of Wu & Su (1990), virus yield using freshly harvested tissue was 18µg/ml or 180µg/Kg and 4µg/ml or 40µg/Kg in case of frozen BBTV-infected banana tissue.

Table 2. Determination of antibody titre through indirect (DAC) and direct (DAS & TAS) ELISA.

Dilutions		Antiserum dilutions (Method II & III)							
Conjug.	**Mabs**	**1:1000**	**1:500**	**1:1500**	**1:2000**	**1:4000**	**1:6000**	**1:8000**	**1:10,000**
1:500	1:500	-	-	-	-	-	-	-	-
1:1000	1:1000	-	-	II+	-	-	III+	-	-
1:1500	1:2000	-	-		-	-	-	-	-
1:2000	1:4000	-	-	-	-	-	-	-	-

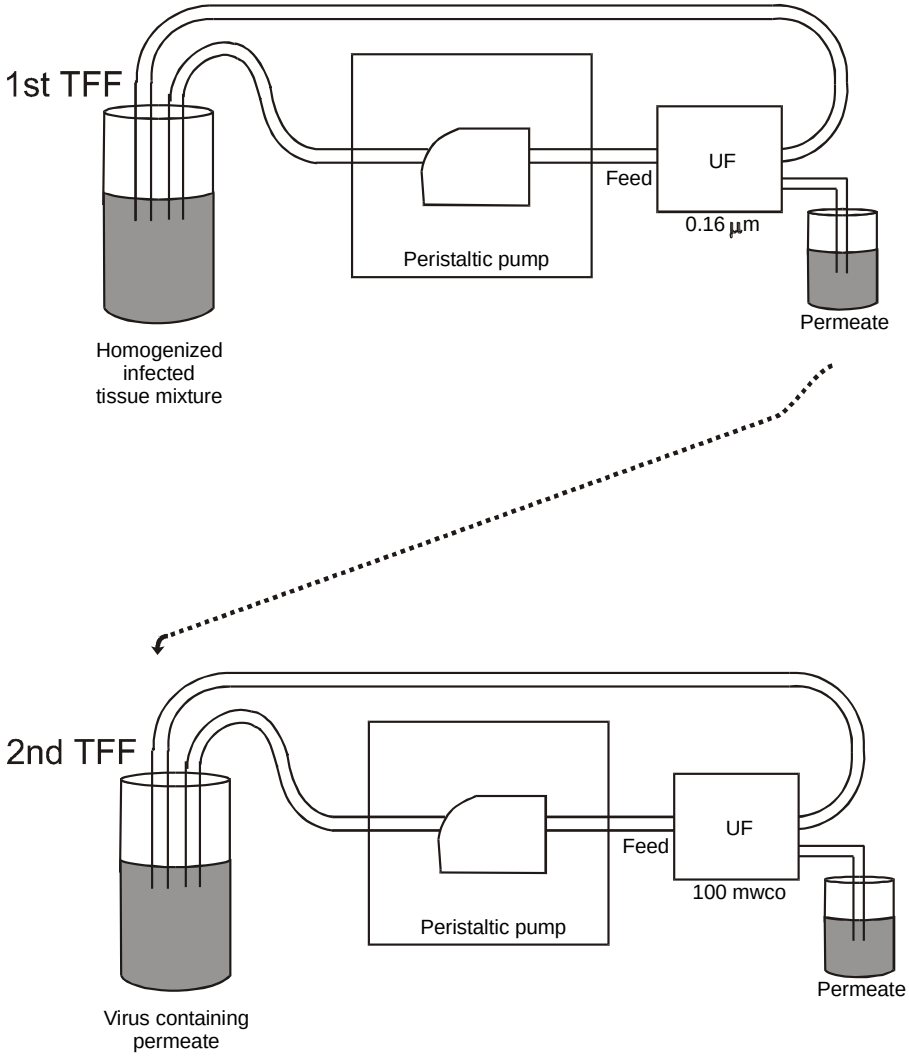

Fig. 3. Ultra-filtration technique employed for BBTV purification.

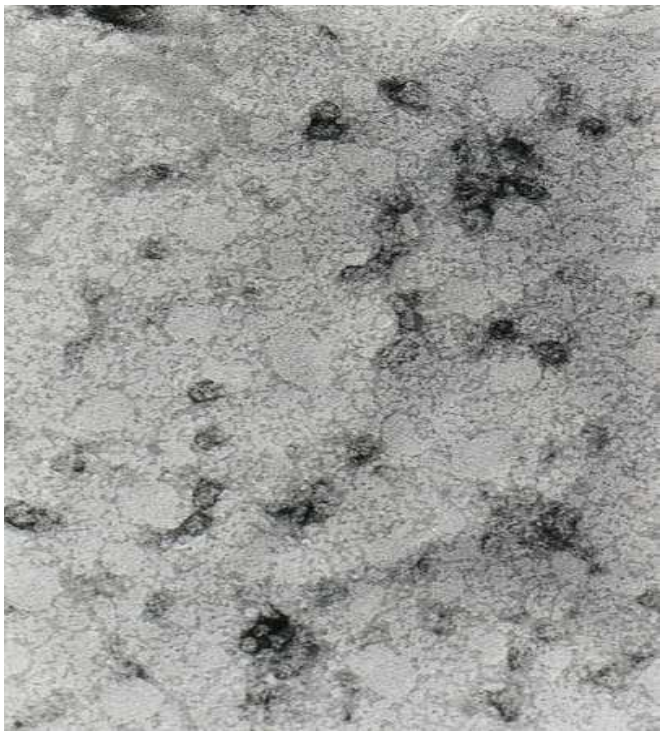

Fig. 4. EM micrographs of BBTV particles in partially purified virus preparation.

Method III: A novel method of Ultra-filtration employed for BBTV purification was tried for the first time and was found successful. EM revealed virions mostly free of host contaminants in purified samples. In permeate and retentate of 1st and 2nd Ultra-filtrations hexagonal isometric particles were observed, while a large number of host contaminants were observed in the rententate. However, no virus particles were observed in permeate of second Ultra-filtration (Fig. 3). The last retentate gave highest absorbance values (0.78, 0.85 & 0.95) over other filtrates. Virus yield in this method was 75µg/ml or 750µg/Kg using fresh diseased tissues. The comparison of two successful methods is given in Table 1.

Serology

Antiserum production and ELISA standardization: The antibody titer of antisera produced by Method II and III was 1:1500 and 1:6000, respectively, as determined by DAC-ELISA (Table 1) and reproduced good results in indirect (DAC) and direct (DAS & TAS) ELISA systems. Extinction Coefficient of the purified IgG (Method III) was 1.3 (approx. 1mg/ml). Antigen and antibodies reacted well at 1:20 (w/v) sap dilution with thousand-fold dilution of enzyme conjugate.

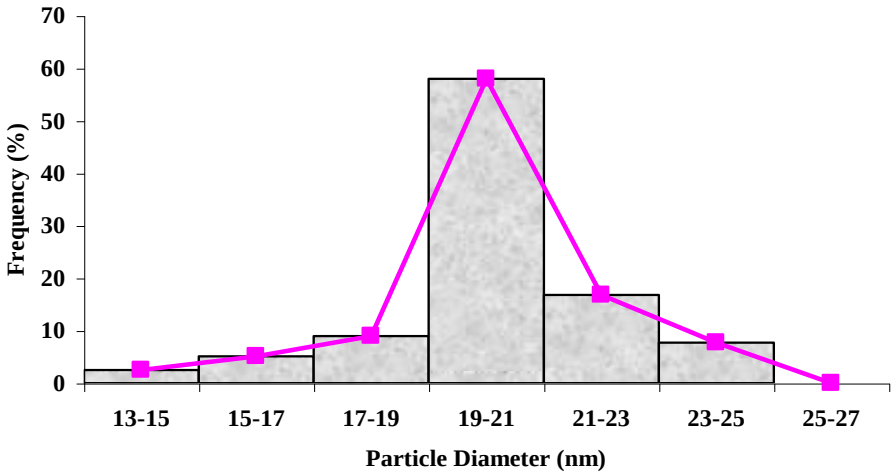

Fig. 5. Histogram showing BBTV particle diameter distribution in partially purified preparation observed under EM.

Discussion

Among the three methods used for BBTV purification, Method II of Wu & Su (1990) was partially successful but Method I adopted by Gold (1961) completely failed in our case. Method III showed better results (4-20 times) as compared to the other two (3.5µg/ml & 18µg/ml) because of high virus yield (75µg/ml) as well as devoid of host contaminants.

In method II homogenizing freshly harvested young infected leaves and midrib preparation gave high virus yield (18µg/ml) as compared to frozen tissue (4µg/ml). The frozen material is difficult to crush which hinder virus extraction on one hand and temperature might have adverse effect on virus recovery on the other hand. Virus particles were observed after differential and density gradient centrifugation but were not found completely free of host contaminants. Most of the virus particles were lost after second Ultra centrifugation as observed under EM, while purification from midrib and petiole preparation gave highest virus yield. This confirms the studies of Wu & Su (1990) and Thomas & Dietzgen (1991). A number of reasons can be attributed to this low yield. This could be that particles were not released from the host cell and lost due to pelleting during clarification step or there could be the differences in pH concentration of extraction and re-suspension buffers, which might have adversely affected the stability of virus particles. Resuspension and stirring of pellets overnight might have degraded virions resulting to virus yield lower than obtained by Wu & Su (1990). Moreover, interference of high content of latex and tannin compounds in banana has been reported to interfere with virus extraction during purification as reported by Dale (1987). These are reports that the virus is strictly phloem-limited and its release from localized cells is difficult due to the abundance of latex, tannin and phenolic compounds of host tissues that hamper virus recovery during purification (Iskra *et al*., 1989; Abdel-Ghaffar 1999; Xie & Hu, 1995; Wanitchakorn *et al*., 1997). However, Thomas & Dietzgen (1991) over come this problem by triturating the

diseased tissue in liquid nitrogen to reduce the interference of these compounds as well as by stirring and incubating the viral extract at low temperature. Due to these impediments, research on this viral disease has been very slow in comparison to other serious viral diseases of banana (Dale *et al*., 1986; Wu, 1987). Furthermore, virus recovery was too low by precipitating the clarified extract with 8-10% polyethylene glycol. The results are consistent with the findings of Thomas & Dietzgen (1991).

Among purification methods, the method of Wu & Su (1990) was successful. Ultra-filtration technique (Method III) yielded good results as compared to conventional method (Method II) of virus purification (Table 2). Moreover, the employed new method skipped most of the analytical steps and saved the time.

The shape of virus particles in purified preparation always appeared hexagonal isometric often having dense electron cores but no coat protein capsomers were evident. The morphology of BBTV particles determined was similar to that reported by Iskra *et al*., (1989), Harding *et al*., (1991) and Thomas & Dietzgen (1991). However, virus particles sometimes appeared ruptured or broken, which might be due to various factors such as deleterious effect of some chemicals/reagents, fluctuation of temperature or over stirring, etc. Staining in 2% UA gave better contrast as compared to PTA. However, virus particles were equally found stable in both stains.

In the present study, method I was not successful which is contradictory to the reports of Gold (1961) because the antibodies produced against healthy host proteins did not bind completely to cross absorb all host proteins in partially purified virus preparation to release virus. In addition, antibodies adsorbed to a solid matrix in a chromatographic column could also facilitate maximum virus recovery, however, it seems difficult.

For substantial quantity of virus purification, enough amount of infected plant material is required. As BBTV is insect vectored, it is difficult to produce sufficient quantities of infected material under controlled conditions as it requires enough time to manage in non-tropical environment (Abdel-Ghaffar, 1999). Overall, previous attempts to purify BBTV showed a low virus yield, as virus particles are phloem-limited. However, different workers have obtained virus yields in various concentrations eg., from 1 Kg of leaves 66-170µg virus yield was obtained by Iskra *et al*., (1989), 640µg by Wu & Su (1990), 300µg by Thomas & Dietzgen (1991) and 0.50-0.52mg/Kg by Abdel-Ghaffar (1999) but in our case, 750µg/Kg virus yield was obtained which is high from previous attempts. It can be further improved using more selective pore size ultra-filters to completely separate virus particles from plant material. Banana plants are difficult to grind and pulverize that can also be improved to release maximum number of virions from host tissues.

The new method of Ultra-filtration was tried for BBTV purification for the first time using three different Millipore-sizes tangential flow filters that retain most host macromolecules. The method appeared more economical, reliable, less time consuming and avoids lengthy analytical steps. The analytical procedures employed by differential centrifugation consume more electricity than this method. Only filters used in this procedure are costly but can be used a number of times. Purification by this method can be completed within 3-4 hours in contrast to routine procedures taking atleast more than 3-4 days. Therefore, this technique can also be adopted for other viruses that are difficult to purify completely from host contaminants by conventional methods.

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