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Full Length Research Paper

Detection of banana streak virus (BSV) Tamil Nadu isolate (India) and its serological relationship with other badna viruses

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Banana streak virus (BSV) is of quarantine significance since *Musa* is a vegetatively propagated crop. Diagnosis by symptomatology is unreliable because the symptoms are variable or absent. Hence, reliable and sensitive diagnostic tests are of major significance. Such sensitive diagnostic tests are also required for virus indexing of germplasm collections. Hence, attempts were made for diagnosis of BSV and to study the serological relationship with other badna viruses. BSV particles were purified from BSV infected plants, collected from the locality of Tamil Nadu, India. Immunosorbent electron microscopy studies revealed bacilliform viral particles with a size of 120 x 30 nm. Polyclonal antiserum raised against BSV reacted with the rice tungro bacilliform virus and sugarcane bacilliform virus in TAS-ELISA. In PCR assays, the primers designed to amplify DNA of BSV Onne isolate amplified DNA of BSV Tamil Nadu isolate producing amplicons of about 644 bp in size. The primers used in PCR to amplify the BSV did not amplify other badna viruses tested such as *Rice tungro bacilliform virus* and *Sugarcane bacilliform virus*. Our results suggest that the BSV isolate from Tamil Nadu is closely related to Nigerian BSV (Onne) isolate.

Key words: Triple Antibody sandwich Enzyme linked immunosorbent Assay (TAS-ELISA), banana streak virus (BSV), polymerase chain reaction (PCR), polyclonal antiserum.

INTRODUCTION

Banana and plantain (*Musa* spp.) are important food sources for millions of people across the globe. Several diseases and pests cause significant yield losses resulting in threatening of the livelihoods of farming community. Among the five virus diseases of banana, disease caused by banana streak virus (BSV) is now occurring in almost all the banana growing areas and has become a major menace to an important commercial banana group 'Mysore' (AAB). Estimated yield losses of between 7 and 90% have been attributed to this disease (Harper et al., 2004; Lockhart et al., 1998). In India, Council of Agricultural Research, Trichy, Tamil Nadu confirmed BSV infection in plants of the cultivar Poovan (AAB). Symptoms of banana streak disease are characterized by conspicuous leaf streaks, which initially are chlorotic and become progressively necrotic (Lockhart, 1986). Foliar symptoms caused by BSV initially resemble those caused by cucumber mosaic virus (CMV) (Jones and Lockhart, 1993). However, necrotic streaks later develop in the leaves of plants infected by BSV, which are not usually seen in the leaves of plants affected by CMV. In addition, BSV-affected plants are stunted and sometimes fail to flower (Frison and Putter, 1989). Even when they flower, bunches are generally very small and poorly filled. BSV is a para-retrovirus member of genus *Badnavirus* belonging to the family *Caulimoviridae* (Selvarajan et al., 2011). Other definitive members of the badnavirus group include: cocao swollen

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shoot virus (CSSV), rice tungro bacilliform virus (RTBV), commelina yellow mottle virus (CaYMV), dioscorea bacilliform virus (DBV), sugarcane bacilliform virus (ScBV), piper yellow mottle virus (PYMV), kalanchoe top spotting virus (KTSV) and schefflera ringspot virus (SRV). A large diversity exists among the virus sources inducing BSV symptoms which are currently subsumed under the species of BSV (Bright Agindotan, 2006).

Five serologically and genetically distinct naturally occurring isolates of BSV have been identified from Costa Rica, Honduras, Morocco, Rwanda and Trinidad. A large genetic diversity of BSV presents a significant problem to virus indexing and to the development of a general diagnostic tool. Hence, attempts have been made for purification and production of polyclonal antisera against the BSV Tamil Nadu isolate. The serological relationship with other badna viruses was studied and presented in this paper.

MATERIALS AND METHODS

Purification of BSV and production of polyclonal antiserum

Infected banana plants cv. Poovan showing typical streak symptoms were collected from the Thondamuthur area of Tamil Nadu. Purification of BSV was carried out as described by Lockhart (1986). The tissues were cut into small pieces of 0.5 cm size and were placed in liquid nitrogen for 1 min and then pulverized. The powder was thawed into two volumes of cold extraction buffer (0.05 M Tris-citrate, pH 7.4, containing 0.5% (w/v) Na_2SO_3, 1% (w/v) polyvinylpyrrolidone (PVP, mol wt. 40,000) and 1% (v/v) Triton X-100). The homogenate was then squeezed through two layers of muslin cloth and clarified by blending for 20 s, with 25% (v/v) chloroform followed by low-speed centrifugation (10,000 *g* for 10 min). The aqueous supernatant phase was collected and the virus concentrated by ultra-centrifugation at 136,000 g for 1 h. The high speed pellets were resuspended in 0.01M phosphate buffer, pH 7.2 and the virus was further purified by centrifugation for 4 to 5 h at 116,000 g in a preformed 0 to 30% CsCl gradient in 10% (w/w) sucrose. The CsCl gradient column was prepared with 0, 10, 20, and 30% CsCl solutions in 10% sucrose solution. It was stored overnight at 4°C before use. 2 ml of purified virus was layered over this and centrifuged at 116,000 g for 4 to 5 h. The virus containing light scattering band was collected with a syringe and dialyzed against 0.01 M phosphate, at pH 7.2 to remove cesium salt. The virus thus collected was stored at 4°C by adding sodium azide (0.01%) and used for serological and electron microscopy studies. Antisera against BSV were prepared in New Zealand white rabbits by intramuscular and subcutaneous injections of purified virus preparations as described by Lockhart (1986). The serum was stored at 4°C after addition of sodium azide (0.01%).

Immunosorbent electron microscopy

Drops of 20 µl of BSV antiserum previously diluted (1:1000) with phosphate buffer (pH 7.0) were placed on parafilm placed in a Petri dish. Formvar coated (200 mesh) copper grids were deposited on the top of each drop so that it can float on the drop. The Petri dish was covered with a lid to prevent evaporation and incubated at 37°C for 15 min. The grid was then washed with phosphate buffer by flooding drop wise from a Pasteur pipette by holding the grid in forceps. Then, the antiserum-coated grids were floated on a 20 µl drop of purified virus preparation on a parafilm. Care was taken to ensure that the antiserum-coated side was in contact with the virus drop and incubated at 4°C overnight. The next day, grids were washed with phosphate buffer and stained with 2% sodium phosphotungstate (pH 7.0). The grids were immediately washed and dried at room temperature. The grids were viewed under JEOL-TEM 100 SX transmission electron microscope.

Detection of BSV infection of plants by TAS-ELISA

Triple Antibody Sandwich ELISA was adopted (Lockhart, 1986). Polystyrene micro plates (Nunc-Immunomaxicorp, Denmark) were used. The plate was coated with polyclonal antiserum diluted 1:5000, produced against Tamil Nadu strain BSV and then samples were added. Samples were ground at a ratio of 1:2 with extraction buffer (pH 7.4) followed by addition of skimmed milk powder (5% in PBST containing 2% polyvinyl pyrollidone). After 2 h, the solution was discarded and BSV monoclonal antibody was added at a dilution of 1:500 µl in PBST (DSMZ, Plant Virus Division, Germany). The rabbit antimouse IgG conjucate (Sigma) was used at a dilution of 1:1000 in PBST. Finally, enzyme substrate 0.6 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) was added to the wells and absorbance at 410 nm (A_{410} nm) was measured using an ELISA plate reader.

Polymerase chain reaction

1 to 2 g of fresh infected and healthy sample was ground in liquid nitrogen. It was transferred to 10 ml of extraction buffer each (incubated at 4°C till all samples were ready for extraction). 1 ml of 20% sodium dodecyl sulphate was added to each tissue. It was kept in a water bath at 65°C for 10 min with swirling. 5 ml of 5 M potassium acetate was added and mixed gently. It was incubated in ice for 20 to 30 min and centrifuged at 5000 rpm for 20 min at 4°C and the supernatant was filtered. 10 ml of ice cold isopropanol was added and mixed gently. It was incubated at -20°C for half an hour. 500 to 700 µl of 1xTE (*high salt TE) was added to dissolve DNA. 7.5 µl of Rnase was used to precipitate DNA 10 µl of 3 M sodium acetate and 2 volume of ice-cold absolute alcohol was added. It was centrifuged at 12,000 rpm for 10 min. The pellet was dried and recovered in TE buffer 100 µl and stored at -20°C.The method used was adopted from Harper et al. (1999). Forward primer BSV 4673 5'-GGAATGAAAGAGCAGGCC–3'; reverse primer BSV 5317 5'-AGTCATTGGGTYCAACCTCTGTCCC–3' were chosen from aligned amino acid sequences corresponding to the AP and RT regions derived from the BSV Onne isolate. PCR cycle condition was an initial denaturation at 94°C for 1 min, followed by 33 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

The presence of virus particles in sucrose gradient fractions (0 to 30% gradients) was confirmed by TAS-ELISA. UV absorption of purified virus preparation showed a peak at 260 nm (Figure 2). The A_{260}/A_{280} absorbance ratio of the virus was 1.13. The virus was not observed in healthy plants. Lockhart (1986) reported that, A_{260}/A_{280} nm of the purified preparation was 1.26. Enzyme immune assays (EIA) established that BSV concentration in infected leaf tissue was related to intensity of symptoms and not to leaf age (Table 3). Markedly increased quantities of virus were extracted in the presence of PVP as reported for citrus tristeza virus

Table 1. Concentration of BSV in different parts of infected plant through TAS-ELISA.

S/N	Parts of infected plant	Absorbance at 405 nm*
1.	Leaf	2.546+
2.	Midrib	2.201+
3.	Petiole	2.324+
4.	Stem	2.124+
5.	Sucker	2.005+
6.	Root	1.987+
7.	Healthy check	0.187-
8.	Blank	0.008

*Mean of eight replications; +, presence of virus; -, absence of virus.

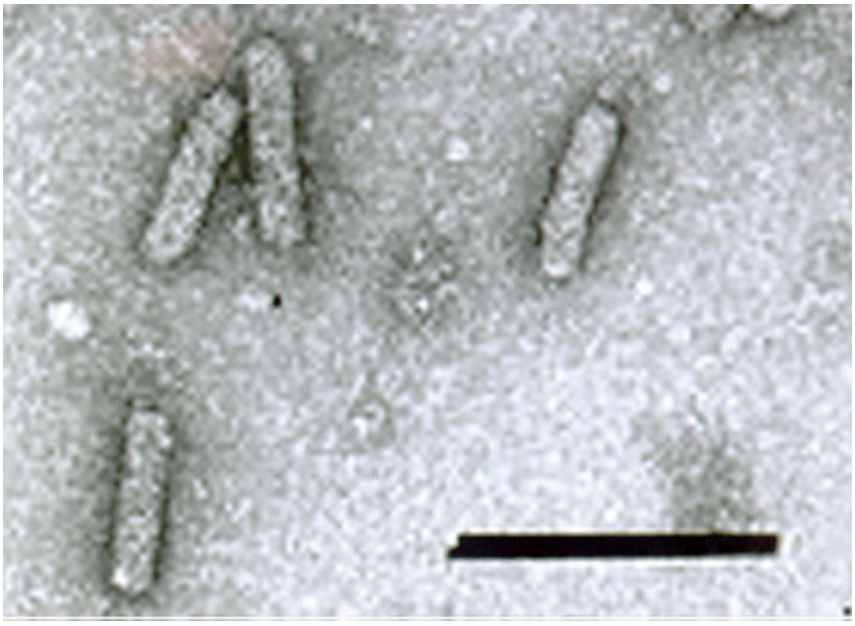

Figure 1. Bacilliform virus particles of 120 x 30 nm.

(Lee et al., 1981) and of Triton X -100. Polyclonal antisera against BSV were produced in rabbits using partially purified virus preparations. The antibodies reacted well in TAS-ELISA test and detected BSV in crude extracts from BSV infected plant parts and not from healthy sap (Table 1). Among the different plant parts infected with BSV, leaf portion had a higher titre of virus (2.546), whereas rhizomes had a lower amount of titre (1.987). Similarly, absorbance at 405 nm for infected leaf was 2.546, whereas healthy leaf sap showed only 1.259 (Table 1).

TAS-ELISA was found to be the best method than DAC-ELISA and DAS-ELISA. Thottappilly et al. (1998) reported that when the substrate incubation time was extended to overnight, the number of samples with A_{405} nm values higher than threshold values almost doubled to about 50%. When different ELISA protocols (DAS-ELISA, ACP-ELISA, PAS-ELISA and TAS-ELISA) were compared for their reliability and sensitivity to detect BSV antigens in leaf extracts, the antisera produced against Tamil Nadu BSV isolate showed specificity in TAS-ELISA only (ELISA plates produced by Nunc-Immunomaxicorp, Denmark was used) which is highly sensitive to TAS-ELISA for the antiserum produced in this laboratory than other ELISA protocols. In other ELISA systems, non-specific background reaction was very high and could not be improved by cross-absorption, choice of buffer, pH or addition of BSA or PVP. In immunosorbent electron microscopy, bacilliform virus particles were detected in purified virus preparations (Figure 1). Viral particles measured approximately 120 x 30 nm in size when negatively stained with 2% sodium phosphotungstate. Through immunosorbent electron microscopy of viral mini preparations, using a multi strain antiserum to BSV is considered as most reliable method for detection of BSV

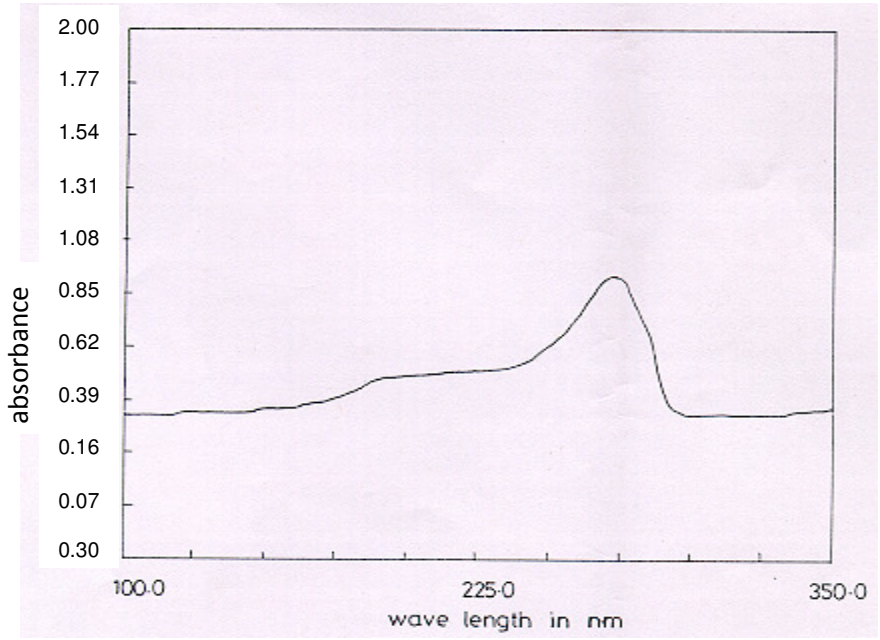

Figure 2. UV absorbance profile of purified BSV preparation.

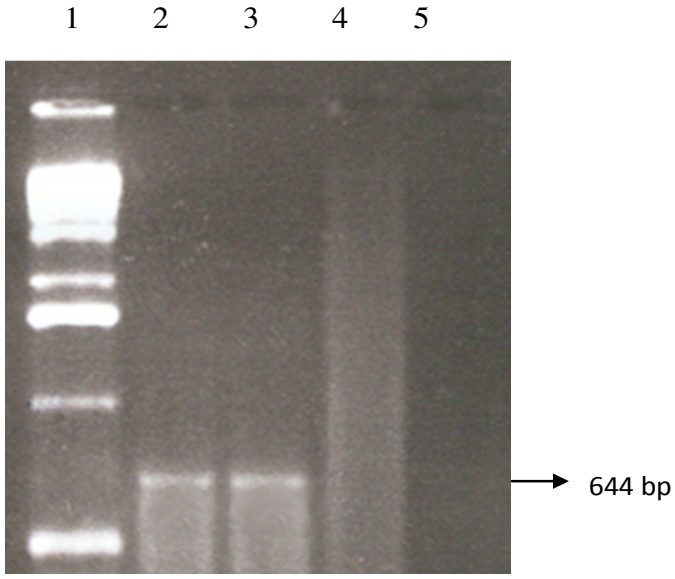

Figure 3. Serological relationship studies by PCR. Lane 1, 1 kb plus DNA ladder; lane 2, purified BSV DNA; lane 3, BSV infected banana leaf; lane 4, RTBV infected leaf; lane 5, SCBV infected leaf.

strains. The limited facility of electron microscopy makes immunosorbent electron microscopy less preferable for routine detection. Therefore, PCR based detection of BSV would be more useful (Anita et al., 2004). As demonstrated in this work, the detection of BSV by PCR is a very reliable, rapid and sensitive. BSV DNA can be detected in sucker well before the symptom appearance. Amplicons of 644 bp were identified in leaves infected by BSV, but not in leaves infected by SCBV and RTBV. In PCR assays, the primers used for the detection of BSV did not amplify the DNA of SCBV and RTBV (Figure 3). BSV primers were chosen from aligned nucleotide sequences encoding for the alanine proline and arginine threonine derived from Onne isolate. Hence BSV Tamil

Table 2. Serological relationship between SCBV, RTBV and BSV through TAS-ELISA.

S/N	Parts of infected plant	Absorbance at 405 nm*
1.	BSV infected leaf	2.208+
2.	SCBV infected leaf	1.857+
3.	RTBV infected leaf	1.973+
4.	Healthy check (BSV)	0.973-
5.	Healthy check (RTBV)	0.673-
6.	Healthy check (SCBV)	0.527-
7.	Blank	0.013

* Mean of eight replications; +, presence of virus; -, absence of virus.

Table 3. BSV concentration correlation with leaf age.

Leaf age	Healthy	Moderately infected	Severely infected
	Absorbance at 405 nm*		
First month after emergence	0.023	1.841	2.578
Second month after emergence	0.014	1.239	2.314
Third month after emergence	0.142	1.457	2.781

Nadu isolate may be closely related to Onne (Nigerian) BSV isolate. Major constraint in BSV detection was periodicity of symptom expression which can be overcome by PCR method. PCR using primers from conserved domain of genomes of badna viruses has been used in numerous studies for rapid, sensitive and reliable detection of different badna viruses (Baranwal et al., 2003). Ndowora et al. (1997) indicate the possible integration of BSV sequences into the *Musa* genome. The integrated sequences do not necessarily lead directly to observable disease symptoms.

Reliable detection of BSV in infected plant materials has become a serious constraint for the safe movement of improved *Musa* germplasm. The results presented in this study, hence demonstrate a PCR-based technique suitable for the large-scale initial screening of *in vitro Musa* germplasm for safer movement.

Antiserum developed against BSV cross reacts with RTBV and SCBV in TAS-ELISA (Table 2). But in PCR assay, primer used to amplify the BSV did not amplify for RTBV and SCBV. Lockhart (1986) studied the serological relationship between BSV, RTBV and SCBV by double diffusion test. SCBV and BSV reacted with each other and produced precipitin lines, revealing that they are serologically related. Lockhart and Autrey (1988) reported that BSV Moroccan (country) isolate and SCBV isolate were found to be serologically related and were initially considered to be the same virus or closely related variants of the same virus. More recent studies have shown that both BSV and SCBV and several other badna viruses are serologically heterogenous and degree of serological relatedness may vary widely among the isolates of a given virus (Lockhart and Olszewski, 1993).

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