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Research Article **Plant Virus**

Detection of *Banana bunchy top virus* and *Banana streak Mysore virus* by PCR: Impact of storing virus infected banana samples

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ABSTRACT

Banana bunchy top virus (BBTV) and *Banana streak Mysore virus* (BSMyV) are economically important DNA viruses infecting of banana in India. A study was conducted to evaluate different methods of storage for BBTV and BSMyV infected banana leaf samples for detection by PCR. Virus infected leaves were stored at room temperature (28°C±2) as dried samples, which were processed by three methods *viz.*, hot air oven at 55°C, calcium chloride ($CaCl_2$), and air drying (28 ± 2°C), at 4°C (in refrigerator) and at –86°C (in deep freezer) as wet samples. BBTV was detected in all samples stored at all conditions up to 15th day of storage. On 30th day of storage, the virus was consistently detected in samples dried in hot air oven and desiccated using $CaCl_2$ but not in samples dried at room temperature. BSMyV was detected in symptomatic leaf samples dried in hot air oven and $CaCl_2$ desiccation on 45th day of storage and even up to 60th day of storage for samples stored at 4°C and –86°C. In case of asymptomatic samples, the virus was detected on 30th day of storage for samples desiccated with $CaCl_2$ and at 4°C and –86°C. Drying the samples by $CaCl_2$ desiccation improved the detection of banana viruses by PCR. It is recommended that the tissue culture industries can dry the banana leaf samples by $CaCl_2$ desiccation before dispatching to accredited laboratories for testing BBTV and BSMyV.

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INTRODUCTION

One of the effective methods for controlling virus diseases in vegetative propagated plants like banana is the use of virus free planting material. For maintaining plants free from viruses, accurate and rapid detection of viral pathogens is essential. Polymerase chain reaction (PCR) has been widely applied for the detection of viruses infecting banana (Thomson and Dietzgen, 1995; Dietzgen *et al.*, 1999; Harper *et al.*, 1999; Manickam *et al.*, 2002; Anitha Cherian *et al.*, 2004; Selvarajan *et al.*, 2007). Normally either fresh leaf tissues or frozen tissues at -86°C have been used for PCR detection and characterization of plant viruses in many crops. The extracted DNA or RNA from infected leaves fixed on to membranes have been stored for considerably long periods and the viruses were successfully detected by PCR (Langridge *et al.*, 1991; Rowhani *et al.*, 1995; Olmos *et al.*, 1996; Singh *et al.*, 2004; Baranwal *et al.*, 2007).

Plant tissues are often difficult to preserve for molecular studies, due to the presence of phenols, polysaccharides and lipids. Denaturation of proteins and degradation of nucleic acids is the greatest obstacle for a successful detection of viruses. Desiccation of plant tissue in Dierite ($CaSO_4$) or silica gel offers the best alternative to collection of fresh or frozen plant tissue (Liston *et al.*, 1990; Chase and Hills, 1991), having been successfully tested by extraction and sequencing of DNA and by digestion with restriction enzymes (Harris, 1993). PCR amplification of DNA from old or poorly preserved tissues can be hindered because various forms of damage reduce the average length of intact template molecules for the polymerase enzyme. Damage to the templates may cause the polymerase to stall, thereby retarding the initial rounds of amplification (Thomas and Paabo, 1994). Freezing temperature lowered the serological values for *Potato virus Y* (PVY) (Stace-Smith and Tremaine, 1970) and *Plum pox virus* (PPV) (Adams, 1978). But it had no

effect in ELISA values obtained with *Apple chlorotic leaf spot virus* (Flegg and Clark, 1979) and PVY (Johnson and Pirone, 1982).

Presently, there are no available reports for stability of DNA viruses infecting banana in dried samples for the purpose of detection by PCR. Fresh leaf samples get rotten during long transit and are not suitable for virus indexing. In the absence of precise information on the effect of storage on detection of BBTV and BSMyV by PCR, the present investigation was undertaken to evaluate the detectability of BBTV and BSMyV by PCR in infected leaf samples stored either in dried form or as wet condition at low temperatures.

MATERIALS AND METHODS

Plant material

BBTV infected hill banana cv. Virupakshi (AAB) and BSMyV infected cv. Poovan (AAB), maintained in insect proof glass house of National Research Centre for Banana (NRCB), Tiruchirapalli were used for this study. BBTV and BSMyV were pure cultured by transferring to healthy plants through aphid (*Pentalonia nigronervosa*) and mealy bug (*Ferrisia virgata*) vectors respectively. Cultivars Virupakshi (AAB) and Poovan (AAB) were chosen because they are highly susceptible for BBTV and BSMyV respectively in India.

Preparation of leaf samples

Fifty gram of leaf samples collected from virus infected banana leaves were stored at 4°C and –86°C as wet storage. Similarly the infected leaf samples were air dried at room temperature (28±2°C), oven dried at 55°C and dried using $CaCl_2$ in desiccators at room temperature. After drying, the samples were packed in air tight screw cap tubes and stored at room temperature throughout the experiment. Each treatment had three replicates and the experiment was repeated twice for confirming the repeatability. DNA was extracted from the stored leaf samples at 15 days interval and detected for the presence of BBTV and BSMyV by PCR up to 60 days as described below.

Isolation of total nucleic acid

Total DNA was isolated following the protocol of Selvarajan *et al.* (2007). Four grams samples were ground using liquid nitrogen to a fine powder and 2 ml of CTAB buffer was added to ground tissues to make a smooth paste. The paste was transferred to a centrifuge tube containing 13 ml of 100mM Tris-HCl, pH 8.0, 50mM EDTA, pH 8.0, 100mM NaCl, 1% 2-mercaptoethanol and 1 ml of 10% SDS. After vigorous mixing, the tube was incubated at 65°C for 20 min. Then 5 ml of 5 M potassium acetate was added and centrifuged at 18000 rpm for 20 min, the pellet was discarded. To the aqueous solution 15 ml of ice cold iso-propanol was added and the mixture was incubated at –20°C for 20 min and centrifuged at 18000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol, air-dried and resuspended in 500 µl of nuclease free water. This DNA preparation was used as template for PCR.

PCR amplification

BBTV: The primers were designed from the coat protein gene sequence of BBTV- Hill banana isolate (Accession no. AY534140). The PCR reaction mix consisted of 1µl template DNA, 2.5 µl of 10X PCR buffer with $MgCl_2$, 1.5 µl of 10mM dNTP mix, 0.5 µl each of BBTV forward (F5'ATGGCTAGGTAT CCGAAGAAATCC3') and reverse primer (R5' TCAAACATGATATGTAATTCTGTTC3'), 1.25 units of *Taq* polymerase (Genei, Bangalore), sterile water to a final volume of 25µl. The PCR reaction was performed in Mastercycler gradient (Eppendorf, Germany) with initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 2 min and a final extension cycle of 72°C for 10 min. A 10 µl aliquot of the PCR product was analyzed on 1% agarose gel at 90V for 1-1.30 hr and bands were visualized and photographed in Alpha Imager (Alpha Inotech Corp., USA).

BSMyV: BSMyV specific primers designed from RT/ RNaseH region of ORF-III as reported by Geering *et al.* (2000). The PCR reaction mixture consisted of 1 µl template DNA, 2.5 µl of 10X PCR buffer with $MgCl_2$, 1.5 µl of 10mM dNTP mix, 0.5 µl each of forward (F5' TAAAAGCACAGCTCAGAACAAACC 3') and reverse primer (R5' CTCCGTGATTTC TTCGTGGTC 3') and 1.25 units of *Taq* polymerase, sterile water to a final volume of 25µl . The PCR profile was programmed to give one cycle of initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min and a final extension cycle of 72°C for 10 min. The PCR amplicons was resolved and visualized as described previously.

RESULTS AND DISCUSSION

BBTV could be detected by PCR in all samples stored with different methods on 15th day of storage (Table 1). PCR amplification of expected size of coat protein gene of BBTV was obtained from oven dried and $CaCl_2$ dried samples on 30th day but not from samples stored at RT after air drying (Fig. 1). Thomson and Henry (1993) used dried leaves of many plants as source of DNA for PCR and RAPD analysis. BBTV was detected in leaf samples stored at -86 °C at 60th day of storage and it could be detected even after one year (data not shown).

All three replicates tested gave uniform results in PCR detection. Thomson and Dietzgen (1995) stored extracted sap in TPS buffer (Tris- HCl, KCl and EDTA) from BBTV infected banana at 4°C for 10 weeks and detected the virus replicase gene by PCR. In our findings the fresh BBTV infected leaf samples could be stored only up to 30 days for positive PCR detection.

In case of leaf samples of cv. Poovan with typical BSMyV symptoms, PCR detected the virus from all samples stored at 4°C, –86 °C, air-dried, oven dried (55°C) and $CaCl_2$ dried samples up to 30th day (Table 2). The amplicon size of 589 bp was observed in PCR in all samples on 30th day of storage by PCR (Fig. 2A).The virus was detected on 45th day of storage in leaf samples that are oven dried and $CaCl_2$ dried but not in air-dried samples. The virus was not detected on further in dried samples. However, BSMyV was detected in samples stored at 4°C and –86°C on 60th day also. Spotted membrane methodology has been successfully used for PCR detection of *Citrus yellow mosaic virus* (CYMV) and greening bacterium and the shelf life of spotted membrane was 60 days (Baranwal *et al.*, 2007). In this investigation we have not tried membrane based spotting and storage for banana viruses. As BSMyV belongs to *Badnavirus* genus, drying of samples looks to be equally better as that of spotted membrane technology for storing and detection by PCR. In the present investigation, BSMyV infected leaf samples could be stored up to 45 days after drying without loosing viral genome for PCR detection which is equally better as that of storing fresh samples at 4°C or –86°C up to 45 days.

In case of asymptomatic leaves of BSMyV infected plants, the virus could be detected in all storage conditions on 15th day. On 30th day, it was detected in samples dried in $CaCl_2$, air dried, kept at 4°C and –86°C but not on oven dried (Table 2; Fig. 2B). BSMyV was further detected in asymptomatic samples stored at -86°C up to 60th day of storage.

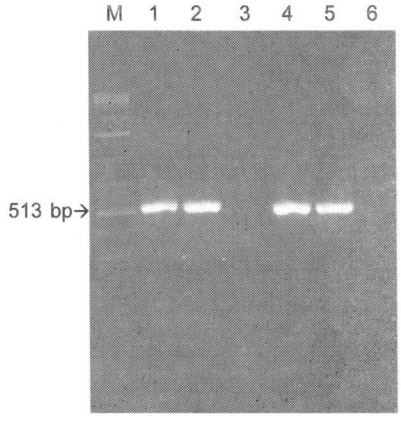

Fig.1: Agarose gel electrophoresis of amplified products of BBTV cp gene from virus infected hill banana leaf samples stored at different conditions on 30th day. M: Marker; lane 1- Oven dried and stored at room temperature, lane 2- $CaCl_2$ dried and stored at RT; lane 3- Air dried and stored at RT; lane 4- Fresh leaf samples directly stored at 4^{0}C; lane 5- Fresh leaf samples stored at –86^{0}C; lane 6- healthy control

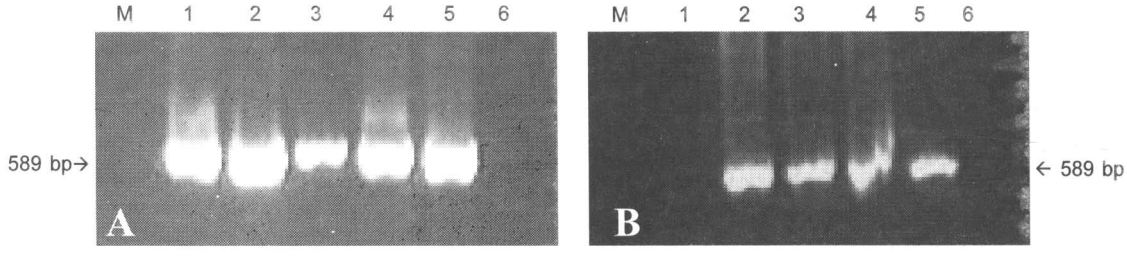

Fig.2: Agarose gel electrophoresis of PCR amplified products obtained from BSMyV infected banana (cv. Mysore) leaf samples stored at different conditions on 30th day. A) Leaf samples obtained from plants exhibiting typical banana streak virus disease symptoms. B) Leaf samples from asymptomatic plants. M- Marker; lane 1- Oven dried and stored at room temperature(RT); lane 2- $CaCl_2$ dried and stored at RT; lane 3- Air dried and stored at RT; lane 4- Fresh leaf samples directly stored at 4^{0}C; lane 5- Fresh leaf samples stored at -86^{0}C; lane 6, healthy control

Table 1. Comparison of different dry and wet methods of storage for PCR detection* of BBTV infected leaf samples of Hill banana

Storage duration (days)	Dry storage			Wet storage	
	Air dried at room temperature	Oven dried at 55°C	$CaCl_2$ dried at room temperature	4°C	–86°C
1	+	+	+	+	+
15	+	+	+	+	+
30	–	+	+	+	+
45	–	–	–	–	+
60	–	–	–	–	+

+ indicates amplification of BBTV in all three samples tested and – indicates non amplification;* each treatment had three replicates and experiment was repeated twice

Table 2. Comparison of different dry and wet methods of storage for PCR detection* of BSMyV infected leaf samples of cv. Poovan (AAB)

Storage duration (days)	Dry storage			Wet storage	
	Air dried at room temperature	Oven dried at 55°C	$CaCl_2$ dried at room temperature	4°C	–86°C
Symptomatic plants					
1	+	+	+	+	+
15	+	+	+	+	+
30	+	+	+	+	+
45	–	+	+	+	+
60	–	–	–	+	+
Asymptomatic plants					
1	+	+	+	+	+
15	+	+	+	+	+
30	+	–	+	+	+
45	–	–	–	–	+
60	–	–	–	–	+

+, indicates amplification of BSMyV in all three samples tested and – indicates non amplification; * each treatment had three replicates and experiment was repeated twice

As maximum of thirty days are required to send samples for long distances for virus testing within India or abroad, more number of infected samples stored up to 30th day under five conditions were tested and the result is furnished in table 3. The result showed that PCR detection was failed in air drying samples for both the viruses. Samples dried using $CaCl_2$ was found superior to oven dried as it showed 100% detection. As for as wet storage is concerned storing at 4°C and –86°C were on *par* with $CaCl_2$ drying.

The present study revealed that storage of oven or $CaCl_2$ dried infected leaf samples up to 30 days which would be useful for detection of viruses, where low-temperatures storage facility like deep freezers are not available. Leaf samples to be indexed can be dried and dispatched to the lab for indexing, instead of sending fresh wet samples, which are spoiled during long transit. Our present results are similar to the finding of Liston *et al.* (1990) and Chase and Hills, (1991) that desiccation of plant tissue in Dierite (CaSO4) or silica gel offers the best alternative to collection of fresh or frozen plant tissue, having been successfully tested by extraction and sequencing of DNA. Best and Gallus (1953) preserved the virus of tomato spotted wilt in dried plant material. For long-term storage beyond 30 days, storage at –86°C was found better for PCR detection of viruses than other methods of storage. Storing samples at –86°C for long time might be useful for virus purification and characterization purpose.

There are no available reports on directly drying the infected samples for the purpose of PCR detection in banana. Singh *et al.* (2004) detected *Potato virus Y* and *Potato leaf roll virus* from extracts immobilized on nitrocellulose membranes stored for more than 65-273 days at room temperature (25°C). Olmos *et al.* (1996) demonstrated print capture PCR on Whatman 3 MM paper or nylon membrane for detection of PPV, such print captured samples could be stored for long time.

Plant tissue samples have been stored at –20°C, after quick freezing at –70°C, with apparently no DNA degradation after 6–8 months (Sytsma *et al.*, 1994). This result corroborates our finding that the BBTV and BSMyV were detectable even on 60th day of storage at –86°C. However, marked degradation has also been observed when leaf tissue stored at –20°C, compared with –70°C (Sytsma *et al.*, 1994). Similar result has been observed also in the present study that the samples of BBTV and BSMyV (asymptomatic) stored at 4°C, degraded faster than samples stored at -86°C. Singh (1983) found that refrigeration of leaves of potato at 4°C or freezing at -70°C preserved the infectivity and serological activity of PVY up to 12 days. Storing grapevine leaf tissue in the refrigerator for 2 to 3 days lowered the sensitivity of ELISA detection; however, freezing expressed sap in glass vials at –20°C gave satisfactory results in case of *Peach rosette mosaic virus* (Ramsdell *et al.*, 1979).

Banana tissue culture industry uses thousands of mother plants for mass propagation. Once the mother plants is extracted from the field for culture initiation, it cannot be kept for more time outside which will lead into contamination at the initiation stage of tissue culture itself. The leaves of mother plant can be dried either at room temperature or in oven or using $CaCl_2$ before dispatching to virus testing labs situated at distance locations. If fresh samples are sent and get delayed during transit, it causes rotting of leaves and makes the tissues not suitable for testing. Further study on validation on storage of DNA viruses along with RNA viruses such as *Banana bract mosaic virus* and *Cucumber mosaic virus* infecting banana are needed for utilizing the technology for storing and indexing of all viruses.

Table 3. PCR detection of BBTV and BSMyV from samples stored for 30 days under different conditions

Storage method	BBTV	BSMyV	
	Symptomatic	Symptomatic	Asymptomatic
Air dried	0/12[a]	1*/10	8/10
Oven dried	11/10	10/10	0/10
$CaCl_2$ dried	12/12	9/9	9/9
4°C	12/12	10/10	10/10
-86°C	11/11	10/10	10/10

* Light band was observed

[a] Number positive /number tested

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