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Molecular Identification of Banana Bunchy Top Virus Associated with Banana Plantation in Uttar Pradesh

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A B S T R A C T

Banana is the most important fruit in India after mango .Its cultivation is affected by various diseases. Among them BBTD (Banana bunchy top disease) caused by BBTV (Banana bunchy top virus) is one of the most important constraints in banana production worldwide. BBTV is a complex circular single stranded DNA virus with multiple genomic components (DNA1-6) and causes heavy economic loss in banana cultivated areas. BBTV is transmitted by its vector (*Pentalonia nigronervosa*). On the basis of symptoms such as green streaks on pseudostem, stunted growth, leaf atrophy, bunchy top appeared on infected plants, leaf samples were taken for isolating genomic DNA using CTAB method with some modification in Selvarajan protocol. DNA was quantified (50ug/lit DNA). PCR amplification was performed on all isolates from jail chungi, Skin center, CCSU Campus, Healthy sample Meerut, field sample no.1 (District Lakhimpur khedi) U.P for early detection of virus. PCR done with the help of specific primers CP (Coat protein gene) and PR(Partial Replicase gene) using total extracted DNA. The Agarose gel was loaded with ladder DNA (1kb), followed by sample DNA. Among DNA samples, all showed amplification by specific primer pair of BBTV. During the study it has been clear that the isolates of U.P were infected by BBTV. So, based on this analysis, it can be concluded that, detection of virus early and proper eradication of infectious plants is very important before passing of virus to another healthy plants.

Introduction

Banana is the most important oldest fruit crop in the word. Its scientific name starts with Genus *Musa* and spp. Paradisiaca, acuminata, balbisiana under Musaceae family. It is significant for its nutritive value with high fibre (0.84%), carbohydrates (22.2%), Vitamins (B6 and C), protein (1.1%) and water (75.7%) with less fat (0.2%).Banana is the second most important fruit crop in India next to mango. it is valued worldwide for its flavor, nutritional value, and availability throughout the year. The first domesticated bananas are thought to have originated somewhere in the Vicinity of New Guinea, Indonesia, the Philippines, or the southeast Asia Peninsula(Perrier *et al.*, 2011) between 7,000 and 10,000 years ago (Denham *et al.*, 2003). Bananas are predominantly produced in Asia Latin

America and Africa. In world leading producers of Banana are Brazil, Ecuador, China, Philippines, Indonesia, Costarica, Mexico, Colombia and Thailand. In India banana production increases around 1 million metric tons from the previous year and the volume of produced accounted 32 million metric tons (During fiscal year 2020).Largest producer of banana in the world is India. In Maharashtra and Gujarat states more than 27% of total banana production takes place. Total area under banana is 3.07 lakh ha. Diseases are the major limitations to banana production and infection occurs by many fungal, bacterial and viral pathogens. Viral diseases are considered to be economically most destructive for banana plantations as they can cause up to 100% crop loss (Amin *et al.,* 2008). Four major viral pathogens, bunchy top virus, streak virus, bract mosaic virus and cucumber mosaic virus are known to cause significant yield loss in India and spread vertically through tissue culture plants.

Banana bunchy top disease was reported in Fiji (1889) and subsequently spread in all other countries. It is one of the most important diseases of banana, causing severe crop losses in many banana growing regions. There is great loss in yield because no fruits develop on infected plants. It also affects some ornamentals such as Canna spp. Banana bunchy top disease (BBTD) is caused by BBTV (Dale 1987), and is classified as a member of nanoviruses based on its molecular characterization (Dugdale *et al.,*1998 and Harding *et al.,* 2000). The vector aphid pentalonia nigronervosa was found to transmit the disease in a persistent manner (Magee, 1940). It is an icosahedral single stranded DNA virus with six genome components identified as the causative agent (Harding, Burns and Dale 1991; Thomas and Dietzgen 1991; Harding et al 1993; Burns, Harding and Dale 1994, 1995) .This BBTV is now recognized as the type member of the genus *Babuvirus* in the family Nanoviridae. BBTV infects plants in the Musaceae family and is a major pathogen of bananas in Hawaii, islands in the south pacific and Southeast Asia. Bananas infected by BBTV show dash like streaks as the first observable symptoms. As the disease progresses, the leaf blades become narrow and the plants show more symptoms such as stunting growth and bunched leaves at the top. The fruits, if any, are malformed. Finally the disease (BBTD) results in the plant death. Due to direct influence on productivity, BBTV is considered to be the most economically destructive disease. BBTV is a phloem-limited virus found in a very low concentration in virus infected plants, which is a major obstacle in the production of specific polyclonal antibodies (PAbs) for its detection.(Dale,1987) recommended a strategy for controlling BBTV based on identifying virus infected plants as early as possible followed by removing the diseased plants. The transmission of BBTV is only possible by vector and through vegetative propagation only, but not by artificial manual inoculation. To reduce the spread of virus, Destroy infected plants with Roundup Ultra Max herbicide.

BBTV is hexapartite, having six different circular single stranded DNA (ssDNA). These six genome components of BBTV are of approximately 1,000 nt long (each component 1kb) and are called DNA-R, DNA-U3, DNA-S, DNA-M, DNA-C, DNA-N(formerly DNA-1 to DNA-6, respectively) (King *et al.,*2012). DNA-R encodes a replication associated protein(rep), DNA-S a capsid protein (cp), DNA-M a movement protein (mp), DNA-C a cell cycle link protein (clink), and DNA-N a nuclear shuttle protein (nsp) genes (Hafner *et al.,*1997b; Aronson *et al.,*2000 ; Wanitchakorn, Harding, and Dale 2000; Wanitchakorn *et al.,* 2000). The function of DNA-U3 is currently unknown.

As this viral disease is emerging as great hindrance in the multiplication and supply of quality planting material both by conventional and in vitro propagation approaches, it is essential to develop a reliable diagnostic method to detect the presence of them in the commercially important varieties of Uttar Pradesh. For the management of viral disease, early detection is the only way as once the plant is infected with virus, there is no cure. There are several reports about the serological and molecular techniques for the detection of viruses associated with banana. However, these techniques are virus

specific and are unable to detect the presence of multiple viruses simultaneously. Therefore, there is a need to use PCR technique for the welfare of mankind. This method is easy, less time consuming and cost effective compared to ELISA. Another advantage is that more number of samples can be handled at a time when compared to other detection methods. As PCR amplifies even very small quantity of DNA sample, this technique is suitable for early diagnosis of the viral diseases. The importance of PCR lies in its ability to amplify specific DNA or cDNA sequences from as short as 50bp to over 10000bp in length more than a million fold in a few hours (Wetzel *et al.*,1991). In this context we conducted molecular identification and evaluation of bbtv in infected plants in the region of Uttar Pradesh.

Materials and Methods

The detection and molecular identification of BBTV in Banana was studied through Molecular and Bioinformatics approaches. Experiment on "Polymerase chain Reaction based detection of Banana bunchy top virus (BBTV)" was carried out in the Department of Genetics and plant breeding (Virology lab) Chaudhary Charan Singh University Meerut.

Plant material and Isolation of genomic DNA from Banana plants

Survey in Meerut, Lakhimpur khedi (U.P, India) was done and leaf samples collected from those areas where BBTD was more prevalent on the basis of morphological symptoms appeared on plants infected by BBTV. samples collected from the plants which showed bunchy top appearance as described by Iskra caruana (1990) The collected samples were stored in the freezer at -80^{0}C (Sanyo Ultra low) . Total genomic DNA from healthy and infected banana leaves was isolated using the CTAB methods with some modification (Selvarajan *et al.*,2002). Briefly, (50-100 mg) leaf samples were grinded in mortar pestle using liquid nitrogen followed by the addition of CTAB (1.5 ml) along with Sarcosil 2 %(200 ul) to make fine paste. This paste was transferred to a pre sterile eppendorf tubes and kept in hot water bath at 60-65^{0}c for 30 min. The mixture was then cooled and centrifuged at 13,000 rpm, for 10 min at 25^{0}C . The supernatant (1ml) was carefully transferred to another fresh tube and equal volume of chloroform: isoamyl alcohol 600µl (24:1) was added and mixed well to get an emulsion by inverting the tube several times for 10 minutes to precipitate sample. Precipitated samples were again centrifuged at 13,000 rpm, 25^{0}c for 10 min. The aqueous phase was transferred into fresh Eppendorf tube (2ml) and 5M sodium acetate (pH 5.2), isopropanol (1ml) was added and then shaked gently for 2 min. After shaking, samples were again centrifuged at 13,000 rpm, temperature 4^{0}c for 10 minutes to settle down DNA Pellate at the bottom of tubes. DNA Pellate was rinsed with 500µl, 70% Ice cooled ethanol and centrifuged at 10000 rpm for 5 min at 4°C.The Pellate was air dried and resuspended in 100ul of Milli Q water without any trace of ethanol present in tube.

Detection of BBTV by PCR

The sequence of the coat protein gene of the viruses BBTV was taken from Gene bank database of NCBI (National Centre for Biotechnology Information). The sequences of these primers and the size of amplicon are listed in the (table 1).all these primers were designed to amplify the fragment of 1kb .PCR reaction was done using 25 ul of reaction mixture containing 2 ul of template DNA, 2.5 ul 10x buffer, 2 ul dNTP mix, 1ul $Mgcl_2$, 2 ul forward , 2 ul reverse primers,1 ul Taq DNA polymerase, 7.5 sterile water. this mixture was set in a Programmed thermal cycler (Thermo fisher scientific Veriti) and PCR was carried out by initial Denaturation of the template DNA at 94^{0}C for 4 min followed by 35 cycles of Denaturation at 94^{0}C for 2 min ,annealing at 52^{0}C for 2 min and extension at 72^{0}C for 2 min with final extension at 72^{0}C for 10 min. the amplified products were analysed by electrophoresis using 1.5% agarose in 1xTAE buffer with Ethidium bromide(5ul) and electrophoresed at 70-80 volt for 30-40 min (Electrophoresis unit Genetix).the gels

were visualized on a UV-transilluminator and gel documentation system(Nugenus syngene system).1 kb DNA Ladder was used as size marker. The PCR amplified products were purified using MinElute gel extraction kit (Thermo scientific).and the purified DNA was stored at -20°C to be send for Sequencing.

Results and Discussion

The presence of BBTV in all samples from the symptomatic plants was confirmed by PCR amplification of about 513 bp and 350 bp products with BBTV CP and PR primer. No amplification was observed in any of the non-symptomatic Banana samples. Total genomic DNA was isolated from all the samples for screening.PCR was set up with banana genome specific primer CP and PR genes. The presence of the bands indicates that the selected samples were infected by viral genome. The result was that, among all six samples collected from jail chungi, skin centre, CCSU campus, field sample no.1 (Lakhimpur kheri) and two healthy samples, four samples showed the presence of BBTV virus after amplification. The size of amplified DNA was 513 bp and 350 bp when compared with ladder. Consequently, the PCR detection method was successful to identify the BBTV infection in plant samples that showed visible bunchy top symptom in Banana plant, whereas the apparently healthy plants did not show any amplification for CP and Rep genes.

Bananas (Musa spp.) are grown as staple food, important cash crops and major export crops in many of the tropical and subtropical areas of the world including India (Annual report INIBAP, 1992). The plants of banana grown at commercial level are basically infertile and are propagated vegetatively, traditionally using suckers. However, recently, for large scale planting of banana, many commercial operators have adopted tissue culture method. Banana is normally affected by four viruses namely, BBTV, BSV, BBrMV, CMV (Burns et al, 1994, Diekmann and Putter, 1996 and Thomas *et al.*,1997). These viruses are radially transmitted through vegetative propagules (Diekmann and putter, 1996) and therefore cause threat to the production. No effective resistance is known in Musa to any of these viruses. So infection can be control by the use of virus free planting material, implementation of quarantine barriers and rouging of infected plants.

Identification of BBTV infection in the suckers is very difficult as the symptom development is not visible at early stage of infection. Immunological detection using ELISA technique is not effective as the virus is present in low tire in the banana plant. Many of the recent literature showed that BBTV detection was effective in using PCR method (Mansoor *et al*., 2005). PCR method had the advantage of amplifying the target nucleic acid present even at very low level and it had become an attractive technique for the diagnosis of much plant viral disease (Henson and French 1993; Xie and Hu 1994; Hafner *et al.*,1997). The leaves of banana plant contains high amount of secondary metabolites, phenolic compounds, which makes difficult to isolate DNA and thereby interfering PCR amplification.

DNA was extracted using Sarcosil chemical and modified CTAB method.PCR screening revealed BBTV infection in four plants and this result was supported by the development of bbtv symptoms. Therefore, PCR based method using CP and rep specific primers was better for the early detection of the disease in banana plants and could be effectively utilized for screening of bunchy top virus.

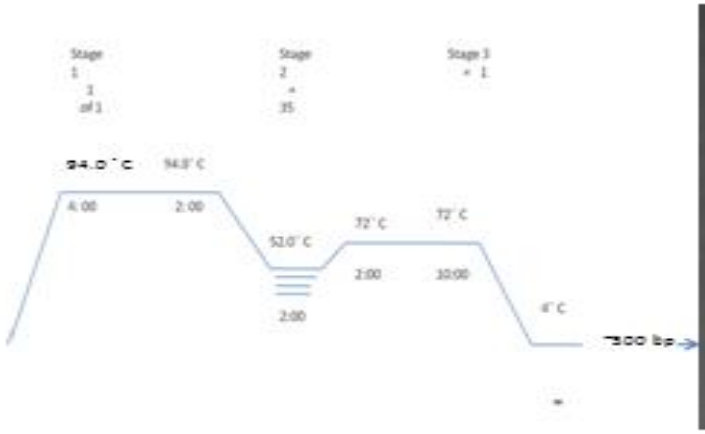
Table.1 Primers used for amplification of different components of BBTV by Polymerase chain reaction

Components	Primer name	Primer sequence (5'-3')	Expected amplicon size (bp approx)	References
DNA-R	BBTV-DNA1-F BBTV- DNA1-R	5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3' 5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3	1110	Harding *et al.*,[21]
DNA-U3	BBTV-DNA2-F BBTV-DNA2-R	5'-GGA CGG ACC GAA ATA CT-3' 5'-ACG TGT CCT ACG AAT TAA-3	1050	Jun and Liu[25]
DNA-S	BBTV-DNA3-F BBTV-DNA3-R	5'-GGT ATT TCG GAT TGA GCC TAC-3 5'-TTG ACG GTG TTT TCA GGA ACC-3'	1058	Furuya *et al.*,[17]
DNA-M	BBTV-DNA4-F BBTV-DNA4-R	5'-CGA TGG CAT TAA CAA CAG AGC GGG TG-3' 5'-CGT TAG GAA CAT AGG TCC AGC GTT TCC-3'	1050	Vishnoi *et al.*,[41]
DNA-C	BBTV-DNA5-F BBTV-DNA5-R	5'-AAC GGA ATA ATA TGA GCT GGC AAC-3' 5'-TAC TGC ATT CTC ACG TGC TGC TGT-3'	1000	Burns *et al.*,[11]
DNA-N	BBTV-DNA6-F BBTV-DNA6-R	5'-GTA TTA GTA ACA GCA ACA AC-3' 5'-TAA CTT CCA TGT CTC TGC TCC-3'	1110	Jun and Liu[25]

Fig.1 Stunted growth of banana plant

Fig.2 Bunched leaves at top with yellowing margin

Fig.3



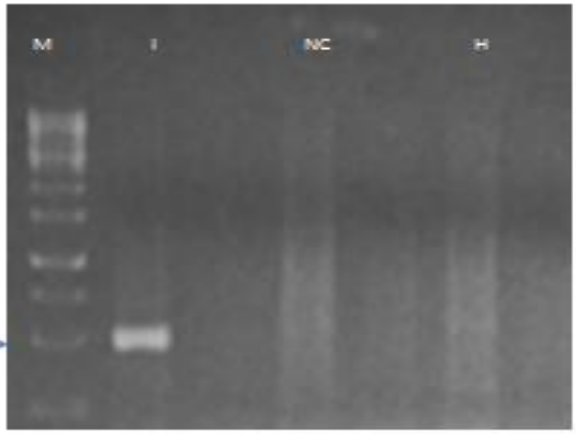
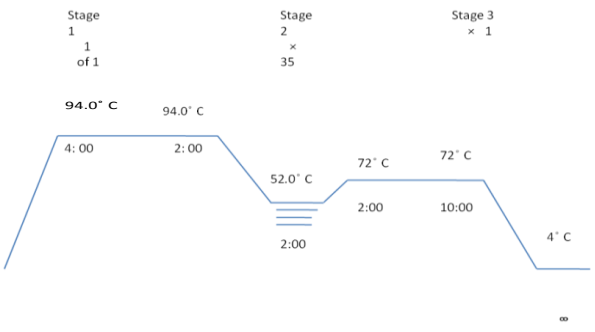


Fig. PCR amplification of infected and healthy samples by BBTV CP primer M: 1 kb DNA marker; I: BBTV infected samples; NC: negative control; H: healthy sample

Fig.4



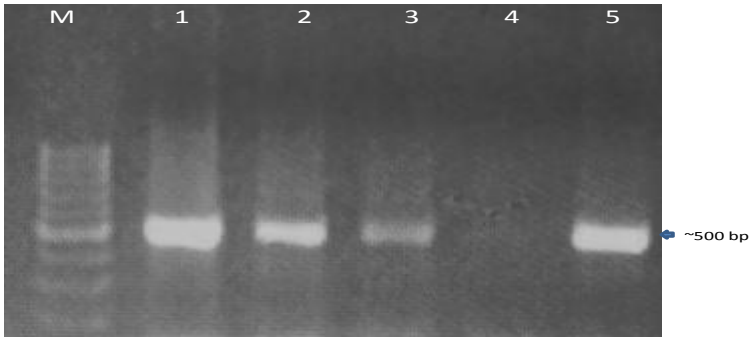


Fig. PCR amplification of infected and healthy samples by primer pair BBTV – CP. M: 1 kb DNA marker; 1: BBTV infected jail chungi sample; 2:Field sample no.1; 3: BBTV infected CCS University sample; 4 :healthy sample; 5: skin center Meerut.

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